



1971

Nuclear Ribonucleic Acid Metabolism and Its Regulation in Regenerating Cutaneous Tissue

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Recommended Citation

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NUCLEAR RIBONUCLEIC ACID METABOLISM AND ITS
REGULATION IN REGENERATING CUTANEOUS TISSUE

by

LEONG-GING WONG

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE
SCHOOL OF LOYOLA UNIVERSITY IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

JUNE, 1971

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ABSTRACT

Abstract of the dissertation entitled Nuclear Ribonucleic Acid Metabolism and Its Regulation in Regenerating Cutaneous Tissue submitted by Leong-Ging Wong in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1971.

Total cellular RNA and the rate of protein synthesis in wound tissue reach a maximal level about 8 days after regeneration begins, and decrease gradually thereafter. Metabolic changes in the cellular nuclei may help unravel the course of metabolic events at different stages of regeneration. Regenerating cutaneous tissue was harvested from rats at short intervals after administration of 5-³H-Uridine. RNA was extracted from isolated nuclei with phenol, and fractionated on ECTEOLA columns. After hydrolysis, the bases in each fraction were separated on Dowex 50 columns. The G+C/A+U ratio was calculated. Radioactivity was measured by liquid scintillation. DNA was determined by the diphenylamine method. RNase activity was assayed by RNA hydrolysis. The level of active and bound RNase was determined. DNA-dependent RNA polymerase was assayed using labeled 5-³H-UTP as the tracer and stripped DNA as the template. Finally, the various types of nuclear histones isolated by dilute acid extraction and acetone precipitation were measured. An attempt to study the mechanisms of gene derepression involved was made by comparing the extent of methylation, phosphorylation and acetylation of various fractions of histones in regenerating

cutaneous tissue with those in regenerating liver. We found that while the amount of cytoplasmic RNA ($\mu\text{M}/\text{mg DNA}$) remains relatively constant, the amount and types of nuclear RNA change. Nuclear RNA reaches a maximal level on the 8th day of regeneration, and subsequently decreases. As wound tissue regeneration progresses, there is a large increase in the proportion of DNA-like RNA in the nuclei. These changes appear to parallel the variations in the rate of protein synthesis observed. A very high percentage of high G+C/A+U ratio RNA, presumably r-RNA, was found in the nucleus of cells in the 5th day wound tissue, indicating that much differentiation of fibroblasts from mesenchymal cells is taking place at this stage of tissue regeneration. The level of DNA-dependent RNA polymerase in the nuclei changes during tissue regeneration; the variations correspond well with the changes in the relative amount of nuclear RNA per cell. The level of active RNase, however, bears an inverse relation to the level of nuclear RNA. It appears that the variations in the level of nuclear and total RNA per cell, and subsequently the protein synthesizing activity in regenerating cutaneous tissue are in part due to changes in the levels of DNA-dependent RNA polymerase and active RNase in the cell. The type of RNase involved is mainly the intracellular alkaline RNase. The activity of RNase in turn is regulated by the amounts of the enzyme synthesized and the RNase-inhibiting-protein present. The relative proportion of the lysine- to arginine-rich histones changes in the regenerating tissue. The mechanisms

of gene derepression in regenerating cutaneous tissue appear to be quite similar to those in regenerating liver. In contrast to regenerating liver, the methylation of lysine-rich histones was readily detected in the regenerating cutaneous tissue. Evidence was also obtained to suggest that during cell replication, the synthesis and accumulation of histones may precede the synthesis of DNA.

LIFE

Leong-Ging Wong was born in Sibul, Sarawak, Malaysia, on September 19, 1941. He graduated from Methodist High School in December, 1960, and received the degree of Bachelor of Science in Chemistry from Baker University, Kansas, in June, 1965.

In September of 1967, he began a program of graduate study in the department of Biochemistry and Biophysics at Loyola University, Hines, Illinois, and was awarded the Master of Science degree in June, 1969.

In February, 1966, he married Polly Tek-Sin Chen. They have one son, Jeffrey Bud-Kin.

He is co-author of the following publications:

1. Metabolism of nuclear RNA in regenerating tissue, Fed. Proc., 29, 317, 1970 (with M.B. Williamson).
2. Changes in the RNA of cellular nuclei during wound tissue regeneration, Proc. Soc. Expt'l. Biol. Med., 136, 1336, 1971 (with M.B. Williamson and C.B. Linsky).
3. Nuclear RNA in regenerating tissue, Fed. Proc., 30, 230, 1971 (with M.B. Williamson and S. Aktipis).

ACKNOWLEDGEMENT

The author wishes to express his grateful appreciation to the late Dr. Martin B. Williamson, under whose guidance these investigations were made. His advice, assistance, encouragement, persistent challenge and inspiration, friendship and zeal for research communicated during this association will long be remembered by the author.

He wishes to express his special thanks to Dr. Maurice V. L'Heureux, who graciously consented to take over the supervision of the writing of this dissertation after the sudden and premature death of Dr. Williamson on November 8, 1970. His assistance, personal sacrifices and warm friendship is deeply appreciated by the author. Thanks must also be given to Dr. Stelios Aktipis, other members of the faculty and the dissertation committee who have helped the author in many respects.

The author also wishes to express his thanks to his wife, Polly Tek-Sin, for her technical and secretarial assistance, and to their son, Jeffrey Bud-Kin, for their encouragement, inspiration, patience and personal sacrifices during the long course of his studies and research.

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Chapter I

INTRODUCTION

Tissue regeneration is a basic and natural reparative phenomenon in mammals which takes place in response to injury. As the term tissue regeneration infers, it involves the production of new cells and the deposition of tissue masses to make up for the tissue deficit in the wound. Hence, during a relatively short time, there occur developmental phases of proliferation and differentiation of cells for specialized functions, and finally the involution of the scar tissue. The study of the biochemistry and mechanism of tissue regeneration, therefore, has important implications on cell biology, as well as great clinical significance.

Although the problems of the healing of wounds have been the subject of study by many for a long time, most of the advances in this area have been made during the last two decades. Early works on tissue regeneration dealt mainly with the description of the physical appearance of the wounds (13, 62, 100, 214, 215, 219, 226, 265). These were then followed by morphological observations at the light microscope level (201, 281). More often than not, they tended to further confuse what was an already murky field. Systematic biochemical studies on the subject have been initiated only relatively recently. The early studies concerned mainly the identification and quantification

of various substances in the connective tissue involved, often with much emphasis on endocrinological effects (214, 226, 265). Owing to the lack of quantitatively precise methods for measuring the rates of tissue regeneration, and the differences between the types of wound involved, much of the usefulness of these early works has been greatly impaired. Nevertheless, a fair amount of biochemical implications can be gleaned from these empirical approaches. In this dissertation, we will deal with the regeneration of the open cutaneous wound only.

Chapter II

REVIEW OF RELATED LITERATURE

ANATOMY OF WOUND HEALING:

Following wounding, a state of acute inflammation develops. Within the first 12 hours an exudate of polymorphonuclear leukocytes, red blood cells, fibrin and macrophages occupies the wound site. Coincident with this outpouring of cellular and plasma elements there is demonstrable increased capillary permeability (49, 215, 287), and swelling and hyalinization of collagen fibers lying within the wound (99, 226), possibly as a consequence of "substances" released in the injured area (55, 145). The polymorphonuclear cell population increases rapidly during the first 24 hours, but these cells undergo fragmentation during the next 48 hours. Whether this fragmentation releases chemotactic substances or not is still unclear. The macrophage population and activity increase between 24 and 72 hours following wounding; and fragmented cellular debris in the wound bed is rapidly ingested (55, 164, 215).

The appearance of fibroblasts can be demonstrated at the periphery of the wound within 24 hours after wounding (66, 84, 90). Most of the recent evidences seem to indicate that fibroblasts in wounds appear to be derived from cells, possibly mesenchymal cells, located in the surrounding connective tissue (84, 90, 219, 258). The mitotic activity of the wound fibro-

blasts becomes very active at about 72 hours after wounding and reaches a peak on the fifth day (84, 100, 281). This rapid increase in the fibroblast population between the third and fifth days signals the end of the first phase in wound healing. Howes, as reviewed by others, has reported that a wound at this stage has no tensile strength (55, 61, 215), but is undergoing active preparation for ultimate fibrogenesis (61).

In addition to the cellular activity described above, mucopolysaccharide appears in the wound within 24 hours after wounding, and reaches a maximum between the third and fifth days. On the basis of histological studies, it was thought that this mucopolysaccharide was formed by the early fibroblast population (26, 149). Following this rise during the first phase, the amount of mucopolysaccharide decreases and the amount of collagen increases as tissue regeneration progresses (61, 77, 262, 270, 286).

From approximately the fifth day to the fifteenth day, the tensile strength of the healing tissue progressively increases as collagen is laid down, and a significant amount of contraction of the wound occurs (13, 55, 212, 283). By the fifteenth to twentieth day, the wound is almost closed, leaving only a little scar. When a wound has regained normal tensile strength, the scar is typically "hypertrophic" and ; histologically, the collagen fibers are of intermediate diameter and organized in

random fashion, and the fibroblast population is still significant (62, 226). Subsequently, beginning some three to six months following wounding, and continuing for a long time thereafter, the scar tissue is gradually replaced by normal skin tissue (55, 214, 265).

PROTEIN METABOLISM IN REGENERATING TISSUE

Tissue regeneration involves not only the production of new cells, but also the synthesis of relatively large amounts of protein (61, 77, 262, 270, 286), particularly collagen (202, 203, 204, 205). Hence for the last few decades, most of the biochemical work on wound healing has been devoted to the determination of the character and the metabolism of proteins by the injured organism.

The most striking change in protein metabolism which results from injury is that, immediately after wounding and for several days thereafter, the animal exhibits a negative nitrogen balance (57, 264, 265). Analysis has shown that there is an increased excretion of urea and all of the amino acids, particularly the essential amino acids. Williamson et al., reported that despite the catabolism taking place following wounding, there appeared to be a retention of sulfur containing amino acids; viz. methionine, cystine and cysteine (265, 266, 267, 269, 271, 272, 279). The first two amino acids were shown to be able to increase the rate of wound healing and to decrease nitrogen excretion in wounded animals. Later, it was reported that the effect of methionine was due to cystine, a metabolic product of methionine, and that methionine was largely converted to cystine (265, 266, 267, 269, 270, 271, 272, 279). Since the predominant protein, collagen, in the regenerating tissue contains no

cystine, it was proposed that perhaps this amino acid is in some way involved in the formation of collagen (265, 266, 267, 269, 271, 272, 279).

The type and content of protein in wound tissue change as tissue regeneration progresses (57, 95, 264, 265, 268). As mentioned earlier, tissue regeneration involves the synthesis of large amounts of protein, particularly collagen. The story of protein metabolism in wound healing, therefore, is essentially one dealing with the structure, synthesis and formation of collagen.

Collagen is an extracellular protein (63, 64). In all known situations in which connective tissue repair takes place, the formation of the precursors of collagen, viz. protocollagens, has been reported to take place in fibroblasts (29, 30, 89, 143, 180, 201, 206). Collagen is characterized by a number of unusual physical-chemical characteristics. It has a unique amino acid composition in that 33% of its amino acid residues are glycine, 11% are alanine and about 13% each of proline and of hydroxyproline (64, 86, 150, 159). It is the only protein in mammals containing significant amounts of hydroxylysine and hydroxyproline (27, 45, 87, 176, 188, 201), a fact that serves as the basis for the determination of the amount of collagen in tissue. It contains neither cystine nor tryptophan residues, and has a tyrosine content of less than one per cent (87, 135,

175, 176). The basic unit in collagen fibers is tropocollagen, which consists of three polypeptide chains, of which two are almost identical (α_1 and α_2) and one is different (α_3) in amino acid composition (58, 92, 165, 176, 177, 178). Each tropocollagen has a molecular weight of about 300,000 (64, 67, 175, 193, 201). Each α -chain therefore has a molecular weight of about 100,000. Although collagen itself is an extracellular protein, it has been shown unequivocally that the precursors of collagen fibers are synthesized intracellularly as are other proteins (23, 29, 45, 111, 140, 185, 228, 231). Despite the presence of hydroxyproline in the molecule, free hydroxyproline, however, is not a precursor of collagen-bound hydroxyproline (3, 140, 175, 185, 188, 201). Hydroxylation of proline to hydroxyproline takes place in the nascent polypeptide chain, and is carried out by the enzyme proline hydroxylase, in the presence of ascorbic acid and oxygen (27, 172, 185). Similarly, hydroxylation of lysine occurs only after lysine has been incorporated into the procollagen (45, 88, 185). It has been demonstrated that hydroxylation of the above amino acids is required before the procollagen can be extruded from the cell (27, 45, 62, 87, 275, 201). The procollagens upon extrusion from the cell form tropocollagen units which are then incorporated into the collagen fibers (175, 176, 180, 281).

In cutaneous wound tissue, no collagen is detectable in the

first two days after wounding. A small but measurable amount of collagen is present in the wound on the third and fourth days (91, 97, 251). Electron microscopic investigations indicate that these early collagens consist of thin fibrils resembling those first found in embryonic development (17). Later, thicker fibrils are found, which appear similar to the collagen fibers in other parts of the body. The total amount of collagen begins to rise sharply on the fifth day, and continues until about the eighth day. Following that, the rate of deposition of collagen decreases (1, 39, 44). The total content of collagen in the regenerating tissue, however, continues to increase slightly until about 15-20 days after wounding (97, 244, 251, 281). Guschlbauer and Williamson (94) studied the change in levels of proteins in the regenerating tissue. They determined the concentrations of tyrosine and hydroxyproline as a measure of cellular proteins and of collagen, respectively. Their results indicated that appreciable deposition of collagen occurred only after the wound tissue has been regenerated for seven days. The content of non-collagenous protein in the wound tissue did not change significantly during the course of the experimental period (95, 108, 280). However, qualitative variation in the non-collagenous protein population were noted (57, 95, 265, 268). Using 5-³H-Proline, Williamson and coworkers, recently studied the rate of synthesis of collagen and non-collagenous protein

during tissue regeneration. They found that the rate of collagen synthesis in the wound tissue increases rapidly from around the fifth day and reaches a maximal rate at about the eighth day. Following that, the rate decreases gradually. However, collagen synthesis still remains at a relatively high rate on the twelfth day. The rate of non-collagenous protein synthesis follows about the same course as collagen. However, its actual rate is much greater than that of collagen (134, 244, 282).

NUCLEIC ACIDS IN REGENERATING TISSUE

The molecular mechanisms regulating the metabolic activities during the regeneration of cutaneous tissue are unknown. Current concepts of cell biology stress the role of nucleic acids in directing cellular functions. In the past, therefore, a number of studies have been conducted by several groups on the various aspects of nucleic acid metabolism in regenerating wound tissue.

The earliest work on nucleic acids in granulation tissue was done on exudates from the wound (131, 148, 274). No definite origin of the RNA in the exudate, however, could be established (60, 215, 216, 221, 274). Tsanev et al., in 1955, reported that nucleotides stimulate the regeneration of wound tissue (251). Subsequent results showed that extensive breakdown of RNA occurred in granulation tissue shortly (24-48 hours) after wounding (186, 248, 249, 250).

In 1961, Williamson et al., studied the total cellular RNA in granulation tissue. They found that after the initial degradative period, the total cellular RNA per mg. of tissue nitrogen increased rapidly and reached a maximal level at about 7-10 days after wounding (274). The amount of total RNA at the peak period was reported to be more than twice as much as DNA in the tissue (96, 108, 274, 281, 282). After the 8th day, they found that the specific yield of total RNA decreased sharply. However, it was pointed out that this latter

phenomenon may be due to the great increase in tissue nitrogen following the first week after wounding (97, 273, 275).

Since total cellular RNA is a mixture of assorted RNA and oligonucleotide molecules, Williamson and co-workers in 1967, undertook to study RNA in the cell nuclei, the site where RNA synthesis takes place. From their study on regenerating tissue harvested on the 8th day after wounding, they reported the isolation of a nuclear RNA fraction purported to be m-RNA, which incorporated large amounts of labeled nucleotides very rapidly (244, 277, 278).

The amount of DNA in granulation tissue increases appreciably as tissue regeneration progresses. However, the rate of formation of DNA is most rapid during the first 4-5 days after wounding, and decreases considerably thereafter (274, 276). Williamson et al., reported that DNA is deposited to a considerable extent before any appreciable amount of collagen accumulation begins (274, 276, 281, 282). Since collagen is synthesized predominantly (if not entirely) by fibroblasts, the above observations indicate that the population of fibroblast in wound tissue increases sharply during tissue regeneration, and that the most rapid rate of replication of fibroblasts occurs at about 4-5 days after wounding. Since the amount of collagen deposited has been used as an index for measuring tissue regeneration, the above findings also explain why there

seems to be an accelerated rate of tissue regeneration beginning at about 4-5 days after wounding.

RIBONUCLEASE AND RIBONUCLEASE-INHIBITING-PROTEIN

There are many detailed reviews dealing with ribonuclease (21, 53, 112, 184). As pointed out by Barnard (21), currently, several types of ribonuclease from a host of living organisms have been identified and extensively studied. Roughly, they fall into two groups; viz. phosphotransferases and phosphodiesterases. The phosphotransferases, as for instance, bovine pancreatic RNase (EC 2.7.7.16), cleave the RNA molecule, yielding 3'-nucleotides; the phosphodiesterases, e.g., snake venom RNase, yield only 5'-nucleotides as the products (21, 70, 133). For our discussion in this dissertation, we refer to ribonuclease to mean only an enzyme specific for RNA.

There are several species of ribonucleases within the aforementioned groups. These are classified on the basis of their pH specificity, relative thermo-stability, and interaction with their inhibitors especially RNase-inhibiting-protein (21, 83, 184, 223, 253). Of these species of RNase, the bovine pancreatic RNase is the best known and most extensively studied. This RNase molecule contains 124 amino acid residues in a single polypeptide chain. It has a molecular weight of around 14,000. The primary structure of RNase has been worked out (116, 195, 217, 230). The RNase molecule contains four intrachain disulfide bridges; viz. between cysteinyl residues 26 and 84, 40 and 95, 58 and 110, 59 and 72 (116, 230, 285). Reduction of these

disulfide bounds, in the presence of 8 M urea, results in total loss of activity as well as changes in the physical and chemical properties of the enzyme (21, 22, 48, 158, 195). The enzyme remains inactive even after removal of the urea. Reoxidation in air at neutral pH, however, reforms the original disulfide bounds, resulting in essentially complete regeneration of enzyme activity and restoration of all the original physical and chemical properties. Based on X-ray diffraction analysis, Wyckoff et al., (285) constructed a three-dimensional model of the RNase molecule. They found that the molecule has an α -helicity of 15%, and that the active site of the enzyme is at the vicinity of histidine residues 12 and 119 which are near each other (78, 284, 285). In mammalian tissue cells, in plants and in some bacteria, the RNase can exist as either active enzyme, or as an inactive latent form conjugated with RNase-inhibiting-protein (83, 139, 227, 229, 253, 254, 255, 256).

The RNase inhibitor was first isolated and described by Roth in rat liver (207, 208). It was shown that the inhibitor was a heat-labile, non-dialyzable substance, readily inactivated by papain, periodate, sulfhydryl reactants and protamine, suggesting that the substance was a glycoprotein (209, 222, 223). Much information has been added since then. The RNase-inhibiting-protein has been found to be active against bovine pancreatic

RNase and alkaline RNases of rat liver, but is inactive against rat liver acid RNase (28, 227). This specificity for certain alkaline RNases is not simply due to the pH of the reaction mixture, since the inhibitor was effective against pancreatic RNase at the pH used for acid RNase assay; i.e., pH 5.8 (223). Inactivation of RNase is brought about by the complexing of the RNase-inhibiting-protein with the enzyme molecule (28, 223, 253). Shortman et al., had shown that the RNase enzyme and the inhibitor-protein are definitely not part of a zymogen system, and that the complexing of the two molecules did not involve the formation of disulfide bonds between them. Since the proteinaceous inhibitor behaved like a polyacid, it was therefore postulated that the inhibitor complexes with the RNase molecule by electrostatic interaction with certain basic groups on the enzyme (222, 223, 227). Experimentally, the bound RNase molecules can be reactivated by treating the complex with Parachloromercuribenzoate (PCMB) or Pb^{++} . PCMB or Pb^{++} inactivates the RNase-inhibiting-protein, hence releasing the RNase molecule (83, 223, 253). The presence of RNase-inhibiting-protein has also been demonstrated in plants and in some bacteria (56, 213, 256).

In vertebrate animals, there are both extracellular and intracellular ribonucleases (21, 200, 217, 254, 256). The extracellular RNases are secreted by the pancreatic and the

salivary glands (21, 192, 200). They have also been found in other body fluids such as milk, urine, semen and serum, where their function, if any, is unknown (10, 192). Pancreatic RNase is generally heat stable; the enzyme remains active even after boiling in water for as long as 5 minutes. The pancreatic enzyme is active at both pH 5.8 and 7.8. However, the pH optimum of the enzyme is 7.8 (21, 48). The activity of pancreatic RNase can be readily inhibited by RNase-inhibiting-protein or heparin at either 5.8 or 7.8 (21, 253). The intracellular RNases include the acid RNase and the alkaline RNase. The acid RNase has a pH optimum of 5.8, and has been established to be one of the lysosomal acid hydrolases (21, 52). It attacks RNA molecules as the pancreatic RNase does, forming 3'-nucleotides as the product (21, 133, 225, 240). Acid RNase does not require Ca^{++} or Mg^{++} for activity (74, 187). In rat liver, the acid RNase is inhibited by heparin, but not by the RNase-inhibiting-protein (223). It is heat labile; the enzyme activity is totally abolished by boiling (21, 223). In plants, the level of acid RNase has been reported to increase strikingly for some hours in response to wounding (56, 213). In contrast, the intracellular alkaline RNase has an optimum pH of 7.8. It is readily inhibited by RNase-inhibiting-protein, but only slightly by heparin (223). Intracellular alkaline RNase is found predominantly in the mitochondrial as well as the cytoplasmic super-

natant fractions of a tissue homogenate (223), and is heat labile (21).

Since RNases degrade RNA molecules, the level of RNases and RNase-inhibiting-protein therefore has a direct effect on the level of RNA in a cell. In regenerating liver, it was reported that the activity of serum ribonuclease decreases after partial hepatectomy. This decrease in activity was found to be due solely to an increase in the level of RNase-inhibiting-protein (253). Similar information in granulation tissue is not available.

DNA DEPENDENT RNA POLYMERASE

RNA polymerase [systematic name: Nucleosidetriphosphate: RNA nucleotidyltransferase (DNA dependent); EC 2.7.7.6] is the enzyme that is directly responsible for the synthesis of all types of cellular RNA. Under the direction of DNA, it catalyzes the sequential assembly of four ribonucleoside triphosphates into RNA molecules (81, 199). The level of RNA polymerase in a cell therefore may be of great importance to the metabolism of nuclear RNA of the cell.

The existence of RNA polymerase was first reported by Weiss et al., (263). It has been found in a wide variety of sources, and exists in all cells in which RNA synthesis occurs (69, 81, 199). Most of the studies conducted, however, were on the bacterial system. Of the few pieces of information obtained from the mammalian system, it appears that the properties of these RNA polymerases are very similar to those isolated from bacteria (16, 79, 81, 190, 199).

RNA polymerase (E. coli) is an acidic protein with an optimal pH of 7.9. Its functional entity is the 13 S unaggregated form which has a molecular weight of 3.7×10^5 (137, 182). The 13 S form in turn is composed of several nonidentical subunits which can be dissociated from each other by urea or detergent. These subunits can then be separated from each other by phosphocellulose, polyacrylamide gels and gel filtration,

respectively, in the above order. Table I shows the distribution and the molecular weights of these subunits of RNA polymerase.

TABLE I
DISTRIBUTION AND MOLECULAR WEIGHTS OF
SUBUNITS OF RNA POLYMERASE (157)

Subunit Designation	Molecular Weight	Subunits per enzyme monomer
α	39,000	2
β, β'	155,000; 165,000	1 ea.
σ or γ	$\sim 90,000$	≤ 1
ω	$\sim 10,000$	(≤ 1)

Subunit σ has been reported to function as the recognition element of the enzyme, involved mainly in initiation of RNA synthesis; subunit ω may not be an obligate constituent of the enzyme (5, 35, 81).

RNA synthesis involves first of all the attachment of RNA polymerase to the DNA, followed by the transcription of the DNA as the enzyme strings along the gene to be copied, and finally, the termination of the transcribing process with the detachment

of the enzyme from the DNA. In the attachment process, RNA polymerase demonstrates strand selectivity towards the DNA. The enzyme shows preference towards the pyrimidine-rich strand, thus ensuring that at one particular segment of the double-stranded DNA, only one of the strands will be transcribed (16, 81, 239). The binding of the enzyme to the DNA strand is very fast, reversible, specific, sensitive to pH and salt concentration, and does not require Mg^{++} (76, 174, 197, 220). The binding is specific in that it binds to a particular region, the initiation site, on the DNA gene segment, which has been postulated to involve a preference of the enzyme for purine nucleotide of pyrimidine-rich strand at the single stranded regions of the DNA (81, 138, 198, 239). Affinity of RNA polymerase to bind with different gene sites on the DNA varies (81, 199). Inhibition of binding of the enzyme to the DNA can be brought about by actinomycin D or proflavin which intercalates between the DNA and enzyme (138, 196). Initiation of RNA synthesis begins with the condensation of nucleoside-5'-triphosphate to the first, generally a purine, nucleotide. The rate of initiation has been reported to be less than the binding rate. It is independent of the concentration of nucleoside-5'-triphosphates, but is dependent upon temperature and the ionic strength of the medium. (174, 197, 199). Initiation of polymerization can be blocked by rifamycin without inhibiting the binding of the

enzyme to DNA (102, 261). In the transcription phase of RNA synthesis, the RNA polymerase strings along the single stranded region, from the 3' to the 5' terminal of the DNA, polymerizing the nucleoside-triphosphates into the RNA chain as dictated by the DNA (81, 199). Rate of transcription by RNA polymerase can be increased by high ionic strength and by addition of ribosomes to the medium. Low ionic strength and low nucleoside-triphosphate concentration will decrease the rate. After transcription has completed, the RNA polymerase detaches from the DNA, perhaps by a termination signal, the nature of which is currently still unclear (199).

Bannai et al., (19), recently studied the activity of RNA polymerase in the normal as well as in regenerating rat liver. They reported that the level of activity of the enzyme in regenerating rat liver was twice as high as that from normal liver. Addition of polycations such as histone and polylysine reduced the level of the enzyme activity in both normal and regenerating rat livers. In comparison, addition of polyanions like polyvinylsulfate in vitro increased the activity of RNA polymerase, perhaps by stripping the histones away from the DNA, exposing more gene sites for attachment of the enzyme. The increase in the activity of the enzyme, however, has been shown to be due mainly to an increase in the level of RNA polymerase and less likely to be due to contribution of the altered status

of the template, or the altered template content, in the nuclei of regenerating rat liver (18, 232). Similar information concerning the status of RNA polymerase in granulation tissue has not been reported to-date.

HISTONES AND GENE REGULATION

In higher organisms, the DNA is covered by chromosomal proteins. The majority of these chromosomal proteins are histones, which are basic proteins containing a high proportion of lysine and arginine and lacking tryptophan. These histones are synthesized in the nucleus of the cell, and can be released from the chromatin by dilute acids or concentrated salt solutions (9, 18, 125, 170, 179, 236, 246). After removal of the histones with acid, the non-histone proteins can be fractionated by alkali which extracts the acidic chromosomal proteins. The remaining proteins in the chromatin are called residual chromosomal proteins (59, 142, 259). It has been shown that when the histones are removed from the DNA, the ability of the DNA to act as a priming template was increased, while adding histones to the DNA decreased the primer activity (8, 236, 246). Hence the current thinking about histones is that they function as gene repressors. Although the non-histone chromosomal proteins may also be involved in gene regulation, very little is known about them. In this discussion, therefore, we will deal mainly with the histones.

Histones are basic proteins with a polycationic nature at physiological pH. There are basically two main groups of histones in the nucleus, the arginine-rich and the lysine-rich histones. The arginine-rich histones can be selectively

isolated from the chromatin by extracting with 0.1 M citric acid, the lysine-rich histones by 0.20 N HCl (8, 18, 125, 179, 246). There are only two major types of arginine-rich histone, having molecular weights of 11,000 and 20,000 (71, 155, 234, 236). They are structurally similar from species to species and from organ to organ (8, 25, 236, 246). De Lange et al., have found that the entire sequence of calf thymus arginine-rich histones are identical to their counterparts from pea pods (54). The arginine-rich histones also contain cysteinyl residues which may be involved in forming complexes by the formation of disulfide bonds with other histones (2, 31, 238). The rate of turnover of the arginine-rich histones is the same as that of DNA. The lysine-rich histones have been reported to be composed of a dozen or more molecular types. Hnilica and co-workers (103, 104, 105) have compared the lysine-rich histones from several tissues and found that there was little difference between them. Unlike the arginine-rich histones, the lysine-rich histones are present in much smaller amounts during the early stages of tissue development. Their proportion, however, increases to the normal level as the tissue matures. The rate of turnover of the lysine-rich histones is much faster than that of DNA or the arginine-rich histones (46, 47, 72, 104, 157, 163). The lysine-rich histones have been reported to associate with regions of DNA rich in A and T, while the arginine-rich histones generally

prefer regions of DNA rich in G and C bases (236). About half of the molecule of each lysine-rich histone is extremely polycationic as would be expected of a powerful DNA binding site, while the other half has a composition similar to non-histone proteins (36, 236).

Currently, there are several theories of gene repression by histones. One of these is the masking theory. Proponents of this theory argue that there are not enough types of histones to match the large number of genes in the DNA. Hence they reason that histones act as gene repressors by simply and indiscriminately covering up the DNA template and thus preventing transcription from taking place (23, 24, 236). The obvious incompatibility of such a theory with the fine degree of selectivity and specificity exhibited by the gene expression mechanism has caused this theory to fall out of favor. Currently, the dominant theory of gene repression by histones is the sequence or site specific theory. According to this theory, each gene has a different and specific repressor. Exponents of this theory reason that the few histones can form different complexes with each other or with other molecules, such as other chromosomal proteins, and in some cases, even with polynucleotides (2, 10, 238). Hence, by permutation, a large number of gene repressors can be formed to match the large number of genes in the DNA (236). Bekhor and others had reported that this was indeed

case in the rat liver (12, 24).

As is obvious from the above discussion, derepression of genes would necessarily then involve the de-coating of the histones from the DNA. DNA-template activity in a cell can be varied by cytoplasmic factors (93, 101, 245) and by hormonal influences (8, 236, 241). Several hormones have been reported to derepress chromatin in the sense that they stimulate the synthesis of nuclear RNA or the production of particular enzymes (20, 50, 125, 237), involving physiological mechanisms which influence the strength and stability of DNA-histone complexes. In recent years, it has been discovered that histones are subject to reactions which modify their structure after completion of the polypeptide chain. The reactions involve group substitutions. Of the few reported so far, they all involved acetylation, methylation or phosphorylation (122, 125, 166, 167, 236). In molecular terms, it has been reported that the acetylation, methylation or the phosphorylation of a histone results in a decrease in the net positive charge on the basic protein. By some yet unexplained mechanism, the de-coated segment of the DNA is then exposed to transcription. Each of the substitution reactions has been found to take place at specific amino acid residue(s) in the polypeptide-chain of a histone molecule. For instance, Allfrey et al., (8), have reported that acetylation of thymus histone always involves only

the lysyl residue at the 16th position of f2a1 arginine-rich histone, although there are eleven lysine residues in f2a1 histone molecule. By amino acid sequence studies, Langan recently reported the finding that derepression of genes following the administration of insulin entails the phosphorylation of a specific serine residue in lysine-rich (f1) histone of rat liver, resulting in an increase in RNA synthesis (125). It has also been reported that methylation of histones occurs mainly at the ϵ -amino or the guanido-group of a specific lysyl or arginyl residue (respectively), of a particular histone molecule (9, 120, 156, 166, 246). These substitution reactions were carried out in the cell by enzymes, presumably under the influence of neuro-hormonal factors (18, 50, 73, 125, 236). For acetylation of the histone, the cell requires acetylase which transfers the acetyl group from acetyl coenzyme A to the histone. Langan and Smith have partially purified a kinase which transfers the phosphate group from ATP to serine residues of histones (120, 162, 167). For methylation via S-adenosyl-methionine, the cell employs methylase which reportedly had also been isolated recently by Langan and others (9, 126). All these substituted groups (viz. acetyl, methyl and phosphate) have been found to turn over much more rapidly than histone (122). Butler found that deacetylation of histones is temperature dependent (37). Inoue and Fujimoto (1969) discovered that it involves an enzyme, a

deacetylase (113). This was followed by the recent exciting report of a deficiency of deacetylase in Novikoff hepatoma by Libby(129). No information is available at this juncture to indicate whether the removal of the substituted methyl and phosphate groups from histones are also enzymatic in nature.

In regenerating rat liver, incorporation of acetate- ^{14}C into the histones occurs in the absence of appreciable amino acid uptake, and is not inhibited by puromycin, indicating that the acetyl groups are attached to histones after completion of the polypeptide chain. In all cases, it was found that acetyl-coenzyme A is the acetyl group donor. The acetylation reaction is reversible and enzymatic, with a Q_{10} of about 2.1 and an activation energy of 12 Kcal/mole (6, 8, 9, 80, 129, 160). Studies with control animals showed that histone metabolism in normal rat liver is characterized by high rates of acetate uptake, followed by a rapid acetyl group turnover. Maximum labeling of the arginine-rich histone was achieved within 15 minutes. The labeled acetate content of the histones fell rapidly after that time, with only 1/3 left after 1 hour (8, 179).

The pattern of acetate up-take and release was strikingly altered in regenerating liver, depending upon the time elapsed after partial hepatectomy. One hour after the operation, an increased acetate uptake was observed. In the period between 1-2 hours after partial hepatectomy, the histones lost only

13.7% of their original acetate content. As regeneration progresses, the turnover of acetate groups resumes (8).

Fractionation of the histones of regenerating rat liver by acetone shows that the highest specific activity appears in the arginine-rich fractions. The peak of acetylation of these fractions occurs between 3 and 4 hours after partial hepatectomy, and falls abruptly thereafter. Characteristically, the acetylation of arginine-rich histones in regenerating liver occurs before the peak of RNA synthesis (246).

The acetylation of lysine-rich histones is not pronounced in normal liver or during the early stages of liver regeneration. At 16 hours, however, labeling of the lysine-rich fraction is readily detectable. In both the arginine-rich and lysine-rich histones acetylation occurs at the NH_2 -terminal amino acids and/or at the ϵ -amino groups of lysine residues, after histone synthesis has been completed (8, 9).

In contrast to histone acetylation, which reaches a peak at 4 hours after partial hepatectomy, the most striking aspect of histone methylation in regenerating liver is its lateness. Tidwell et al., had shown that there was very little methylation of histones taking place during the first 16-20 hours after the operation (246). The rate of methylation peaks sharply about 30 hours and then declines abruptly. All the methylation was found to occur at the ϵ -amino group of lysine residues of

arginine-rich histones, in the form of either ϵ -N-methyllysines or ϵ -N-dimethyllysines, or both. However, ϵ -N-dimethyllysine is the predominant form of the modified amino acid residue in the arginine-rich histones (120, 166). It appears that only one amino acid residue per histone molecule is methylated (8, 236). The methylation of arginine-rich histones involves enzymes which had been isolated from the nuclei of regenerating liver (9, 120). No lysine-rich histones were found to be methylated in the regenerating rat liver (246). So far the biological effects of histone methylation in liver regeneration has not been deciphered. It has been found that the methylation does not correlate with an increase in DNA template activity for RNA synthesis, nor with the initiation of histone, non-histone protein, or DNA synthesis. It was inferred by Tidwell et al., that it may correlate with the structural and functional changes known to occur in the nucleus prior to mitosis, which involve a condensation of the chromatin and a curtailment of nucleic acid synthesis (246). No information is available to-date concerning the phosphorylation of histone, if indeed it occurs at all, in the regenerating rat liver.

Biochemical similarities as well as wide differences exist between the metabolism of regenerating cutaneous tissue and that of regenerating liver. No information is available to-date about histones and gene-derepression in cutaneous wound tissue.

However, since the above described gene derepression mechanism seems to be a universal one in higher organisms, it is thus hoped that the above review of the gene derepression in regenerating liver may serve in some way as a guide to our study of the same problem in regenerating cutaneous tissue.

Chapter III

THE PROPOSED STUDY

The molecular mechanisms regulating cellular activities during the regeneration of cutaneous tissue are unknown. Present-day concepts of cellular biology emphasize the role of nucleic acids in directing cellular activities and functions. The necessary information for metabolic processes is contained in the nucleotide sequences of deoxyribonucleic acid (DNA). Complementary ribonucleic acids (RNA) carry this information to the cytoplasmic sites of protein synthesis. In order to better understand tissue regeneration, such as the variation in the rate of protein synthesis observed at different stages of wound healing, we will study the metabolism of RNA in cell nuclei of regenerating wound tissue.

The first phase of the study will involve the isolation and identification of RNA types (groups) in the nuclei, as well as the measurement of the amount of nuclear RNA, with particular emphasis on each type of RNA, at those stages of tissue regeneration where dramatic changes in protein synthesis (or tissue regeneration) occurred. At the same time, we will study the rates of synthesis and turnover of these different types of nuclear RNA. In order to get a better picture of RNA metabolism during tissue regeneration, we will also study some of the factors affecting the synthesis and catabolism of RNA in granu-

lation tissue. Hence, in the second phase of our study, we will investigate the level of ribonuclease and DNA-dependent RNA polymerase in the wound tissue as regeneration progresses. In the third phase of our study, an attempt will be made to follow the mechanism of gene derepression in regenerating wound tissue cell nuclei.

To study the metabolism of nuclear RNA of wound tissue at different stages of regeneration, groups of rats will be given standardized wounds in the scapular region, and maintained on a protein-free diet. To synchronize with the stages during which great changes in the rate of protein synthesis has been observed, the regenerating tissues will be harvested on the 5th, 8th and 12th days after wounding. For studying the rates of incorporation and turnover of various nuclear RNA molecules, the animals will be given 5-³H-Uridine at different time intervals before collection of the wound tissue.

For the studies on nuclear RNA, the regenerating tissue will be homogenized in buffered sucrose solution, and the nuclei isolated by gradient centrifugation. The different types of RNA will be extracted from the lysed nuclei by aqueous phenol extraction at neutral and alkaline pH. In the preliminary study, absorption spectra of the RNA extracts will be checked for any qualitative changes in the RNA population during tissue regeneration. For further identification, and hence a more accurate

study of any difference in RNA population during tissue regeneration, each nuclear RNA extract will be fractionated on an ECTEOLA cellulose column. After mild alkaline hydrolysis, the constituent nucleotides will be separated on a Dowex cation exchange column, and the concentrations determined spectrophotometrically.

The rate of synthesis and turnover of each fraction of nuclear RNA will be assessed by liquid scintillation counting of the level of radioactivity in the RNA. The amount of DNA in each tissue sample will be determined by reaction with diphenylamine. The relative yield of total and each type of nuclear RNA during tissue regeneration will then be computed.

To measure the level of ribonuclease activity in the wound tissue at different stages of regeneration, the regenerating tissue samples harvested on the 5th, 8th and 12th days after wounding will be homogenized in the cold. After centrifugation, aliquots of the supernatant will be assayed for ribonuclease activity using yeast RNA as the substrate. Following incubation for a set time interval, the reaction of the enzyme mixture will be stopped, and the undigested RNA substrate precipitated by the addition of acid-ethanol. Optical density of the digested RNA will be a measurement of the relative level of active ribonuclease in the tissue. To measure the amount of total (ie. active and inactive) ribonuclease in the tissue, the supernatant

obtained after centrifugation will be treated with lead acetate, and then assayed for the enzyme as described above.

To assess the level of DNA dependent RNA polymerase in regenerating wound tissue, nuclei will be isolated from the wound tissue homogenates as described above. The amount of RNA polymerase in the lysed nuclei will be assayed using 5-³H-uridine triphosphate as the tracer. To guard against any possible variation in the template activity of wound tissue at different stages of regeneration, aliquots of pooled liver DNA will be used as the template for all the assays. After incubation, the enzyme reaction will be stopped, and the newly synthesized RNA precipitated by the addition of acid ethanol. Specific activity of the synthesized RNA will be taken as a measurement of the relative amount of RNA polymerase in the tissue.

Finally, in the third phase of our study, an attempt will be made to follow the modes of gene de-repression involved which resulted in the pattern(s) of RNA metabolism in regenerating wound tissue. To accomplish this, groups of wounded animals will be injected with (CT₃)-methionine, CT₃-C(=O)-O⁻ or ³²PO₄³⁻, and the wound tissue harvested as described above, on the 5th, 8th and 12th days after wounding. After homogenization, the lysine-rich histones will be extracted from the isolated nuclei with 0.1 M citric acid and the arginine-rich histones will be isolated with dilute HCl. The amount of histone and radioactivity in each

extract will be measured to give an indication of the degree and type of mechanism of gene derepression involved in the wound tissue at different stages of regeneration.

Chapter IV

MATERIALS AND METHODS

In this chapter, we will describe in detail the experimental procedures employed in the study of nuclear RNA in regenerating tissue as outlined above.

NUCLEAR RNA METABOLISM DURING TISSUE REGENERATION

Animal Specification and Care:

Virgin female rats of Sprague-Dawley strain were used in all the experiments. To ensure that they could survive the trauma of wounding, only rats weighing 200 ± 20 gms at the beginning of the experiment were used. To guard against possible variations in metabolism arising from seasonal changes and differences in population compositions, all of the 240 rats involved in this series of experiments were purchased from the same animal breeder at one time. The rats were housed in individual cages at one location under the same environmental conditions. Water, and food prior to wounding, was allowed ad libitum (266, 270, 272, 278). At this stage, the rats were fed regular rat chow (Taklad Incorporated, Monmouth, Illinois).

Wounding Of Animals:

After a period of stabilization for 2 to 3 days in individual cages, the animals were anesthetized by the subcutaneous administration of 25 mg. of sodium pentobarbital per kilogram of body weight. The rats were then kept in the cages for 20 to

30 minutes, until they succumbed completely to the anesthesia. The hair in the scapular region of the rat was clipped and the outline of a 4.0 cm. diameter disc was traced. The skin of the marked area was then excised (244, 266, 272). The wound was then gently blotted with cotton wetted with alcohol. Generally, no bleeding or very little was encountered. By this method, close to 100% survival of the animals was achieved. Subsequent to wounding, the animals were given a protein-free diet (Nutritional Biochemicals Corporation, Cleveland, Ohio) as in previous studies in wound healing from this laboratory (281, 282, 283). To obtain uniform dietary intake, each rat was allowed 11 gms of food per day (264, 265, 271, 278), which was generally completely consumed.

Harvesting Of Wound Tissue:

To study the metabolism of nuclear RNA during tissue regeneration, experiments were conducted on the wound tissue from three groups of 80 rats each, harvested on the 5th, 8th and 12th day, respectively after wounding.

Prior to harvesting of the regenerating wound tissue the wounded rats were weighed and their physical condition was noted. The animals exhibited loss of weight, scraggy and rough fur. To study the rate of turnover of various types of nuclear RNA, each animal was injected subcutaneously with 118 μCi of $5\text{-}^3\text{H}$ -Uridine in 0.05 M phosphate buffer at pH 7.3. At intervals

of 15, 30, 45 and 60 minutes after the administration of labeled uridine, the regenerating wound tissue from the rats, in groups of 20 animals at a time, were harvested. The clotted fibrin scab was removed, and the wound was scrubbed clean. The regenerating wound tissue was then carefully excised without including any underlying tissue (266, 272, 273). Any blood on the excised tissue was blotted at once. The harvested wound tissue was then immediately frozen in liquid nitrogen to prevent degradation of the RNA. Frozen tissue samples were stored individually in air-tight polyethylene bags at -18°C until extraction procedures could be carried out (265, 271). To guard against possible diurnal metabolic variations, the wounding of all animals and the harvesting of the regenerating tissue were carried out at approximately the same time of the day, between 10.00 A.M. and 2.00 P.M.

Isolation of Nuclei:

Regenerated wound tissue is composed mainly of tough connective tissues, the bulk of which are collagen fibers. Embedded and wrapped in this mass of fibers are a small number of cells, the majority of which are fibroblasts. To study the metabolism of nuclear RNA, it is necessary, first at all, to isolate the nuclei from the cells in the regenerating wound tissue. Any contamination of the nuclear sample, such as the presence of whole cells due to incomplete isolation of the nuclei, must be

avoided. For these reasons, the separation of the nuclei from the cells in the regenerating wound tissue has been one of the most critical steps of the experiment. All operations up to the end of the phenol extraction step were carried out at between 0° - 4° C to prevent the degradation of RNA molecules.

The pooled frozen tissues from 20 rats were first weighed. In lots of about 5 gm. each, they were then rendered brittle by further chilling in liquid nitrogen. The tissues were pulverized in a steel mortar pre-chilled with dry ice, by repeated pounding with a hammer. This step is necessary for breaking the tough connective collagen fibers in the tissue sample. The pulverized tissue was next transferred to a 16 x 150 mm pyrex, Potter-Elvehjem homogenizer, and to it was added 8 ml. of cold 0.25 M sucrose solution containing 0.003 M CaCl_2 prepared in 0.05 M of Trishydroxymethylaminomethane (Tris) buffer at pH 7.2. With the pestle of the homogenizer attached to a drill press, the pulverized sample was homogenized for about 10 minutes (266, 272, 273, 274, 278).

The homogenate was suspended in 40 ml. of the same cold sucrose solution and shaken for one hour. The suspension was centrifuged at 700 g for 45 minutes. The supernatant, as well as the cellular debris on top of it, was discarded. The residue which contained collagen fibers, nuclei and some unbroken cells (41, 218, 266), was resuspended in 5 ml. of sucrose solution

and further homogenized for 5 minutes. The homogenate was again shaken in 30 ml. of sucrose solution for 45 minutes, followed by centrifugation at 700 g for 45 minutes. The supernatant was decanted. Histological smears of the residue were made. These were then stained with hematoxylin and eosin, and examined under a light microscope. As was reported by Thachet (244, 298), the residue contained intact and some broken nuclei, together with large amounts of collagen fibers. The presence of collagen in the preparation is of no great concern, since the method employed for the extraction of ribonucleic acid will readily exclude it from the extracts. No whole cells and essentially no cytoplasmic material could be detected in the isolated nuclear material.

Aqueous Phenol Extraction of RNA:

There are many and voluminous reviews which describe the various methods employed for the extraction of RNA from tissues (112A, 121, 121A, 122A). However, currently, the preferred and most extensively used procedure is that of aqueous phenol extraction, first described by Kirby, Sibatani and others (82, 121, 224). Many different variations of the technic have since then been devised, each having its own unique applicability (51, 33A, 112A, 121A). Basically, however, all the extraction procedures depend on the partition of RNA between aqueous buffer and phenol. The main advantages of this approach are that it yields relative

ly large amounts of undisrupted RNA molecules, and that it separates RNA from DNA and protein (75, 82, 121A).

In our experiments, the isolated nuclei in the precipitates were lysed by the addition of 2.0 ml. of 2% sodium lauryl sulfate (244). The detergent also inhibited any ribonuclease present and hence facilitated the isolation of intact RNA molecules (33, 82). The ruptured nuclei were suspended in 20.0 ml. of cold 0.05 M Tris buffer at pH 7.2. To the suspension was added 20.0 ml. of 88% freshly distilled phenol. After shaking vigorously for one hour in the cold, the mixture was centrifuged at 25,000 g for 45 minutes. The aqueous layer at the top was carefully siphoned off with a syringe, and the RNA in it was precipitated by the addition of 100 ml. of 95% cold ethanol and 3 drops of 10% NaCl solution (244, 278). Three more extractions were carried out on the phenolic residue with 20.0 ml. portions of Tris buffer at pH 7.2. The aqueous phase containing the RNA obtained from each extraction was precipitated separately in 95% ethanol spiked with 10% NaCl. In a preliminary study, the relative amount and types of nuclear RNA isolated by successive aqueous phenol extractions was measured. The pertinent data are presented in Table II. Owing to the small amount of nuclear RNA in Extract 4 as shown in Table II, for better and easier analytical purposes it was combined with Extract 3. After the 4th extraction, the phenolic emulsion was

TABLE II
RELATIVE AMOUNT OF NUCLEAR RNA ISOLATED BY
SUCCESSIVE AQUEOUS PHENOL EXTRACTIONS

Extracts	Extraction at pH	% yield	G+C/A+U
1	7.2	56.8	1.5
2	7.2	19.1	9.8
3	7.2	4.3	5.0
4	7.2	7.9	6.9
5	7.2	1.8	0.7
6	8.6	5.0	0.4
7	8.6	1.1	0.9
8	8.6	0.7	0.6
9	8.6	3.2	0.8

extracted with 20.0 ml. of 0.05 M Tris buffer at pH 8.6, in the same manner as with the neutral buffer. Three extractions were carried out at pH 8.6. Each RNA extract was again separately precipitated with cold 95% ethanol spiked with sodium chloride. Again, because of the small yield, Extracts 7 and 6 were combined. About 5% of the total nuclear RNA remained in the phenolic residue which could be extracted only with KOH solutions of such high concentration that considerable degradation of the RNA occurred (244). Hence, no further extraction with pH higher than 8.6 was carried out. After standing overnight in the cold to allow for complete precipitation, the RNA precipitate was centrifuged at 700 g for 30 minutes. The RNA obtained was resuspended in a volume of distilled water, freeze-dried and stored in the cold.

As shown in Table II, the RNA in each extract as isolated by the above aqueous phenol extraction procedure appears to be different on the basis of their G+C/A+U ratios. Hence it was employed as a primary fractionation step in our study of metabolism of nuclear RNA in regenerating wound tissue.

To get some indications whether there is any qualitative change, and if so, what type of change there is, in nuclear RNA at different stages of tissue regeneration, a preliminary study was made by comparing the absorption spectra of the nuclear RNA extracted from regenerating wound tissue. Each RNA extract was

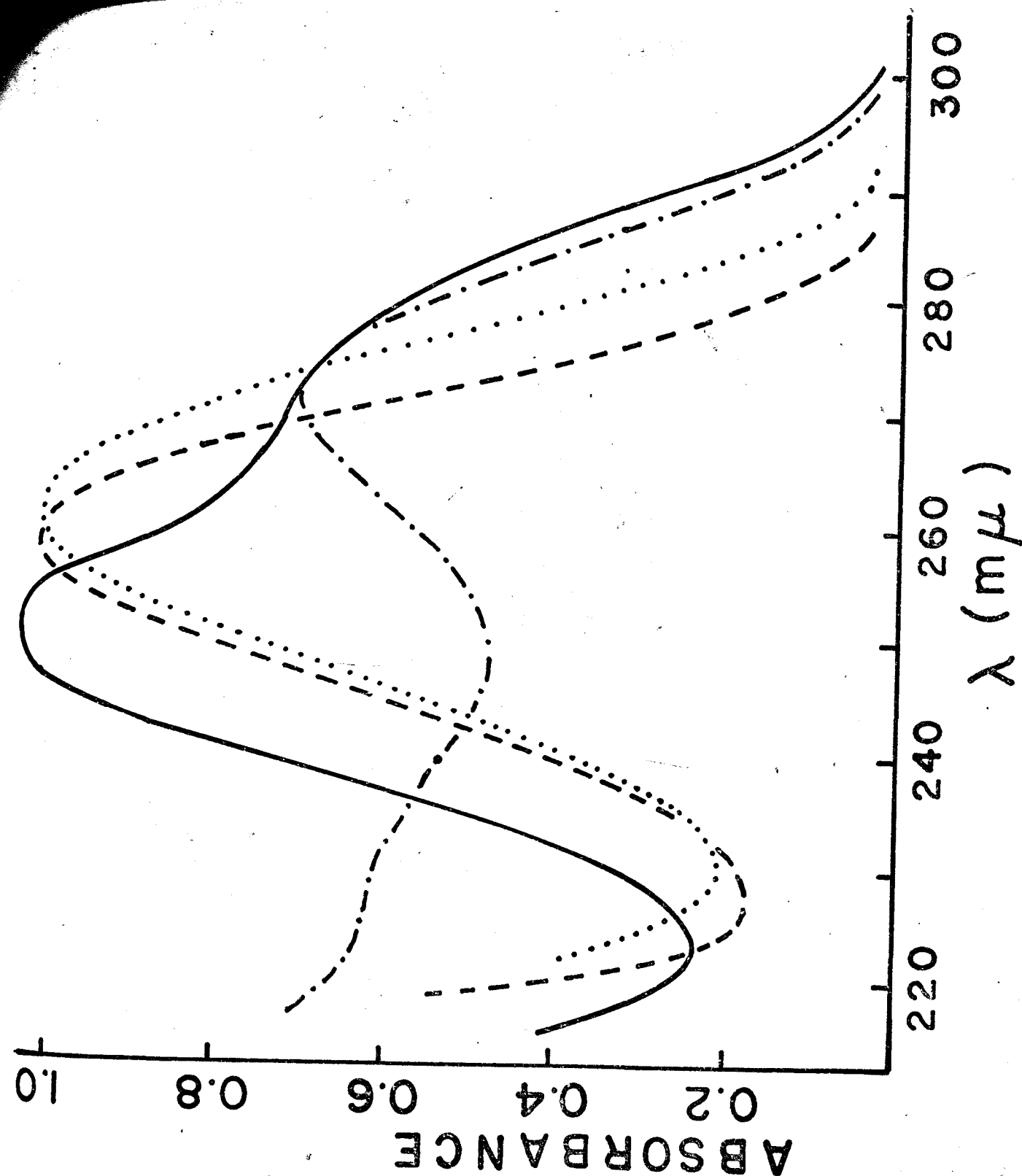
dissolved in 0.05 M, pH 7.2 Tris buffer. The absorption spectrum of each extract between 230 and 300 nm was then recorded on a Cary 15 spectrophotometer. RNA in each extract was then recovered by cold ethanol precipitation; followed by centrifugation at 700 g.

By superimposing the absorption spectrum of the same RNA extract from various stages of regenerating wound tissue, a comparative study of the nuclear RNA populations at different stages of tissue regeneration was made. Any gross change in the spectrum can be taken to indicate a change in the base composition of the RNA; hence a change in the make-up of the RNA population of the extract. Each RNA extract in turn was analyzed by referring to the spectra of the four pure nucleotides at pH 7.2 (Figure 1) to give some indications as to the nature of change, if any, which occurred in the RNA in the nucleus during tissue regeneration.

Fractionation of RNA:

There are a number of methods available for the fractionation of RNA; e.g. gradient centrifugation (136, 260), and chromatography on DEAE cellulose (168, 169, 173, 233, 242), Sephadex (15, 107, 118, 151, 286), hydroxyapatite (82, 132, 135A), methylated albumin (68, 114, 152, 183) or ECTEOLA cellulose columns (233, 244, 278). Each of these procedures has its particular adaptability as well as drawback(s). One problem

Fig. 1: Spectra of nucleotides at pH 7.2. (---) represents the spectrum of cytidine monophosphate (CMP); (—), Guanosine monophosphate (GMP); (....), Uridine monophosphate (UMP); (- - -), Adenosine monophosphate (AMP).



common to all these methods of fractionation is that they do not necessarily result in the isolation of a pure species of RNA. In this laboratory, use of ECTEOLA cellulose columns has been found to give the best resolution for nuclear RNA and with consistent results. The type of RNA eluted from the ECTEOLA column will depend to a certain extent on the concentration of the LiCl solution used.

ECTEOLA cellulose for the column was prepared by suspending, with gentle shaking, 4 to 5 gm. of the anion exchanger (medium mesh, 0.4 mg/gm capacity) in 300 ml. of 0.05 M Tris buffer at pH 7.2. The mixture was allowed to stand for 20 minutes, and the cloudy supernatant was discarded. The cellulose derivative was resuspended in 300 ml. of the same buffer and washed a second time. The resultant ECTEOLA cellulose in a thick slurry was poured into a funnel attached to a 30.0 x 1.0 cm. column with a sintered glass disc at one end. The glass column was then filled with Tris buffer, and the cellulose was allowed to settle by gravity. After it was packed for 3 to 4 hours, the column was washed by running through it about 500 ml. of the 0.05 M Tris buffer. The settled ECTEOLA cellulose bed was 18 cm. in height. The flow rate through the column was regulated to be about 30 ml. per hour.

Each nuclear RNA extract was dissolved in 5.0 ml. of 0.05 M Tris buffer at pH 7.2. The sample was then loaded onto the

ECTEOA column which had been connected to a 0.3 ml. capacity flow cell placed in the sample holder of a Cary 15 spectrophotometer. Fractionation of the loaded RNA sample was achieved by eluting with 0.2 M, 0.4 M and 0.6 M LiCl in 0.05 M Tris buffer at pH 7.2, and 1.0 M LiCl in 0.05 M KOH, respectively. The whole process of elution was continuously monitored at a wavelength of 260 nm. Four RNA fractions were obtained from each extract of nuclear RNA. Further elution by higher concentrations of LiCl and KOH did not yield any more RNA fractions.

The separated RNA fractions of each extract were collected individually and precipitated in cold 95% ethanol spiked with 10% NaCl as described above.

Hydrolysis of RNA:

To determine the base composition of the RNA fractions so that the ratios of nucleotide bases could be calculated, a reliable method of hydrolysis of the RNA samples was required. Various methods of hydrolysis were tried. Yeast RNA was suspended in 6 N HCl and heated in a boiling water bath for one hour. The resultant hydrolysate when passed through a Dowex 50W-X4 cation exchange column and monitored at 260 nm with a Cary 15 spectrophotometer showed only one broad peak on the chart. It was later found that hydrolysis with 6 N HCl caused loss of purines (51, 211). Hydrolysis with 1.0 N NaOH caused deamination of some bases, especially of nucleotides of cytidylic acid, as

well as loss of some adenylic and uridylic acid (4, 75, 123, 124, 153, 154).

In the method finally adopted, the RNA precipitate was suspended in 0.5 to 1.0 ml. of 0.1 N NaOH (75, 123, 141, 153).. The tube containing the suspension was sealed with parafilm and maintained at 37°C for 18 hours, with occasional shaking. The hydrolysate was neutralized with HCl and flash evaporated to dryness. The validity of this procedure of hydrolysis of RNA is discussed under the next heading.

Separation And UV Spectroscopy of Nucleotides of RNA:

To identify a RNA molecular fraction, the base composition had to be determined. Seraidarian and Steiner (235) had made use of the differences in absorbancy of nucleotides at various pH values and used the absorbancy of characteristic wavelengths to determine the approximate base compositions of mixtures (119, 247). Reid and Pratt (191) and later, Vasilenka and Lee et al., (127, 257) have used computers to resolve the spectra of nucleic acid hydrolysates into their components. Guschlbauer et al., (94) and Pratt et al. (181) using linear programming have claimed that the base composition can be determined exactly, and in the latter case, even without recourse to hydrolysis.

Claims have also been made by a group in China about solving the base composition problem with four simultaneous equations (132). As will be evident in the next paragraph, these

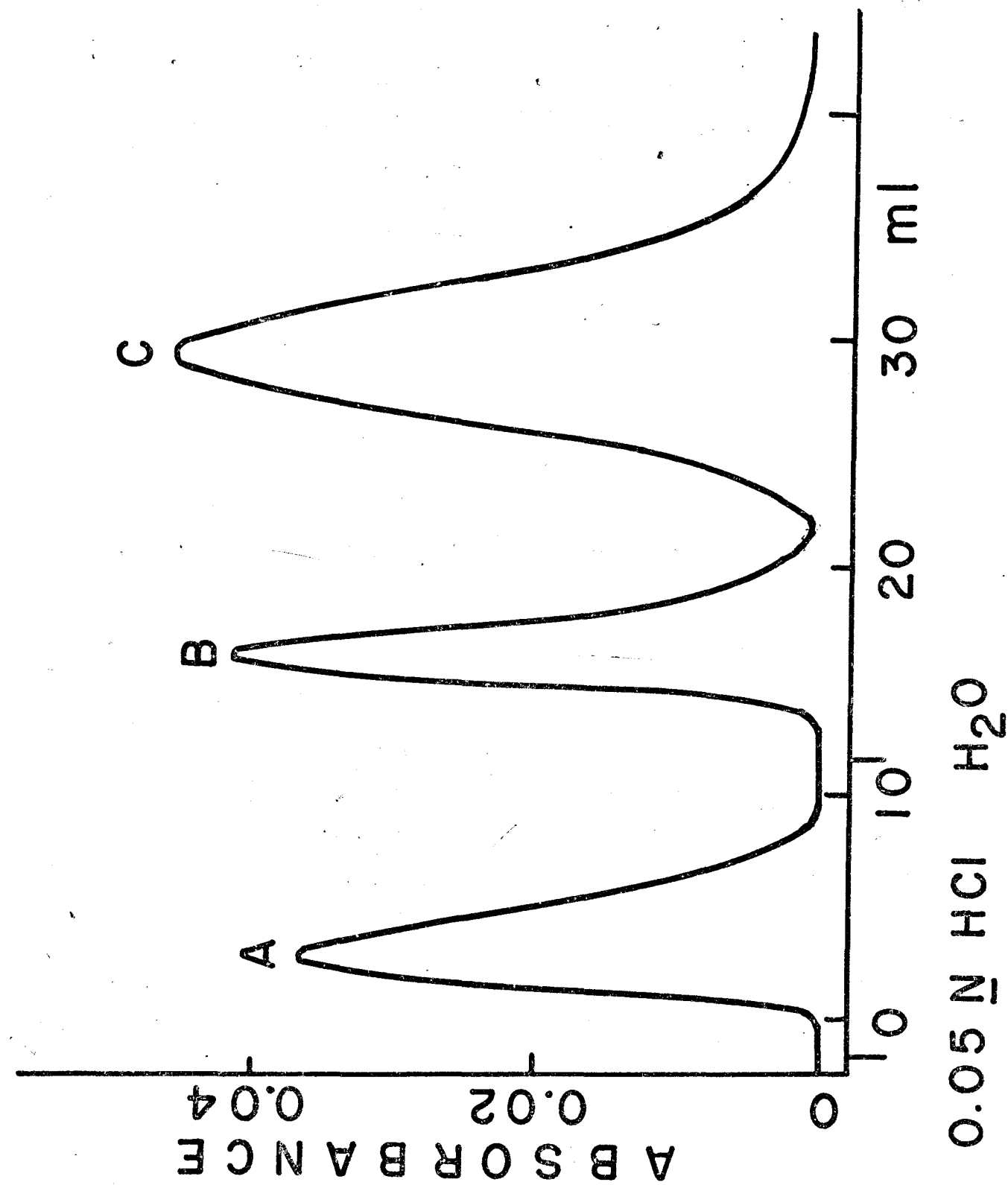
methods have either proved to be unreliable or totally erroneous. Several of these methods were tried in this laboratory. Under the best of conditions mixtures of pure nucleotides could be measured with 1 or 2% error, and at worst with 30 to 40 % error, of the actual base composition of the mixture. Even the linear programming method which made use of a library of spectra of different ratios of the four nucleotides has been admitted by Pratt to be applicable only to certain special cases (181, 194). It then turns out that the only reliable method is to measure the individual nucleotides after separation from each other.

The method finally adopted to separate the nucleotides was a modification of that developed by Katz and Comb (117). About 5 gm. of Dowex 50W-X4 resin, (200 to 400 mesh) was suspended in 100 ml. of deionized water. The resin was degassed and allowed to equilibrate for 2 to 3 hours with gentle stirring. After decanting most of the supernatant, the slurry was poured into a 1x12 cm. glass column. The settled resin bed was 7 cm. in height. With the reservoir attached, the column was washed with about 300 ml. of deionized H_2O . It was then treated with 200 ml. of 0.05 N HCl. The nucleotide mixture from each hydrolyzed fraction was dissolved in 1.0 ml. of 0.05 N HCl and loaded onto the column which was attached to a 0.3 ml. capacity Beckman flow cell in a Cary 15 spectrophotometer. The column was first eluted with 0.05 N HCl and was continuously scanned at 260 nm.

The chart speed on the recorder was set to run at one division per 2 minutes; the flow rate of the column was regulated to be not more than 1.0 ml. per minute. The first 9.5 ml. of the eluate collected contained all the uridine monophosphate loaded on the column. After this, all the guanosine monophosphate was eluted and contained in the first 7.5 ml. of the H₂O effluent. The next 25.0 ml. of the H₂O effluent contained both cytidine monophosphate and adenosine monophosphate. The pattern of elution is shown in Fig. 2. With the absorbance switch of the Cary 15 set to read 0.10 full scale, this Dowex 50W-X4 column was able to resolve a sample of RNA as little as 5 to 10 µg. This method was found to be well suited to analyze the meager RNA samples extracted from regenerating tissue.

Nucleic acids absorb light in the far ultra-violet region. This property is inherent to the purine and pyrimidine bases (147, 181, 191). Generally, in assessing the concentration of an RNA sample, one measures the optical density at 260 nm. However, very often one encounters a hypochromic effect in the presence of secondary structures of RNA (51). The hypochromicity is a function of various energy transitions arising from interactions between the bases (147), and may cause a decrease in absorbance of up to 35%. Upon hydrolysis, the hypochromicity disappears due to release of restriction on electron orbitals of the bases imposed by the polynucleotide structures (85, 106,

Fig. 2: Elution profile of nuclear RNA hydrolysate on Dowex 50W-X4 column. Fraction A = UMP, B = GMP, C = AMP & CMP.



146, 252). Hence prior to the determination of the concentration of the nucleotides, the RNA fractions separated by the ECTEOLA column were subjected to complete hydrolysis.

Acid and base catalyze the hydrolysis of RNA in the pH range of 1 to 13 (4, 75). Above pH 12, only 2'- and 3'-phosphates are formed (154). At moderate to high salt concentration, RNA bases exhibit stacking effects which profoundly influence the rate and degree of hydrolysis of RNA (124, 189). This effect is negligible at pH 13 and at an ionic strength of 0.1. Accordingly, the RNA fractions obtained from the ECTEOLA column were hydrolyzed with 0.5 ml. of 0.1 N NaOH.

Different bases, and their nucleosides and nucleotides, have different characteristic absorption spectra. At pH 1, maximal absorption of 3'-AMP, 3'-UMP, 3'-GMP and 3'-CMP occurs at 257, 260, 257 and 279 nm, respectively (83A). The proximity of the wavelengths at which maximal absorbance occurs creates serious spectral anomalies due to the overlapping of the absorption bands (65). Hence it is well nigh impossible to resolve the spectral data of a mixture of nucleotides into its individual components (115).

To measure the base composition of the RNA fractions, individual nucleotides were separated by the Dowex 50W-X4 column as described above. The identity of each nucleotide was verified by its absorption spectrum of 0.05 N HCl. The amount of each nucleotide isolated was calculated from the absorbance at the

λ_{max} . in 0.05 N HCl. The UMP nucleotides obtained from the nuclear RNA fractions were saved for the determination of radioactivity after the absorbance was measured.

The measurement of GMP and UMP were made directly from the respective standard curves of absorbance vs. nucleotide concentrations (Fig. 24 and 25 in appendix). The absorption spectrum of pure AMP overlaps that of CMP considerably. Hence in a mixed spectrum of AMP and CMP, the total optical densities at 257 and 279 nm cannot be taken as the optical densities of pure AMP and CMP, respectively. As indicated before, these two nucleotides were eluted together from the Dowex 50W-X4 column. However, in this case, the maximal absorption wavelengths of the two nucleotides are sufficiently far apart that the respective maximal absorbances can be resolved by the following simultaneous equations:

$$A_{257} = A_{257, \text{AMP}} + A_{257, \text{CMP}}$$

$$A_{279} = A_{279, \text{AMP}} + A_{279, \text{CMP}}$$

where:-

A_{257} = Total absorbance of mixture at 257 nm

$A_{257, \text{AMP}}$ = Absorbance at AMP in mixture at 257 nm

$A_{257, \text{CMP}}$ = Absorbance of CMP in mixture at 257 nm

A_{279} = Total absorbance of mixture at 279 nm

$A_{279, \text{AMP}}$ = Absorbance of AMP in mixture at 279 nm

$A_{279, \text{CMP}}$ = Absorbance of CMP in mixture at 279 nm

Solving the two simultaneous equations, we get

$$A_{257,AMP} = \frac{2.32(A_{257}) - A_{279}}{2.08}$$

$$A_{279,CMP} = A_{279} - 0.238(A_{257,AMP})$$

From the calculated absorbances, the corresponding concentrations of AMP and CMP can be determined from the respective standard curves of absorbance vs. nucleotide concentrations (Fig. 26 and 27, in appendix).

DNA Determination:

To compare the level of RNA in wound tissues at different stages of regeneration, it is necessary to compute the specific yield of RNA in terms of some stable frame of reference. This problem was approached by studying the DNA content of the wound tissue, which is fairly constant per cell and is directly proportional to the number of cells in the tissue.

The DNA content of the wound tissue was determined by the diphenylamine method (75, 154). It was found that the DNA content of the isolated nuclei was not affected by the extraction of the RNA with phenol. An aliquot of the isolated nuclei, as described above, was suspended in 5.0 ml. of 2% citric acid. Another aliquot of isolated nuclei was used for the extraction of RNA with phenol (7 Extractions). The DNA in the residual

phenol was precipitated in 50 ml. of anhydrous ether. After centrifugation, the precipitate was washed twice with 30 ml. of ether and dried. The DNA content of both the nuclei suspension and the ether precipitate was assessed as described in the next paragraph.

To a weighed aliquot of ether precipitate was added 5.0 ml. of 2% citric acid and 2.0 ml. of 20% trichloroacetic acid. The suspension were then placed in a water bath at 90°C for 30 minutes, with occasional stirring. This treatment hydrolyzed the nucleic acids to their nucleotides and precipitated the proteins. The hydrolysates were then centrifuged at 2,500 rpm for 30 minutes. The supernatant was pipetted off. Into a set of test tubes were added 2.0 ml. of water and 2.0 ml. of the supernatants, respectively. 4.0 ml. of diphenylamine reagent consisting of 1.0 gm. of diphenylamine dissolved in 100.0 ml. of glacial acetic acid and 1.75 ml. of concentrated sulfuric acid was then added to each of the tubes. The tubes were next placed in a boiling water bath for 10 minutes with occasional stirring. A blue color was detected in the sample containing DNA nucleotides. Diphenylamine is specific for the deoxyribose moiety of the nucleotides; the presence of any RNA nucleotides is of no concern. After cooling, the absorbance of the samples were determined at 600 nm in a Beckman DU spectrophotometer, using the water sample as the blank. The concentration of DNA

of the solution was then evaluated from a previously prepared standard curve of absorbance vs. known DNA concentrations (Fig. 3). From the weight of the tissue sample, the specific yield of DNA (mg. DNA/ unit tissue weight) was assessed. Specific yields of RNA of wound tissue at different stages of regeneration was computed as unit RNA per unit weight of DNA.

Data in Table III indicates that there is no loss of DNA during the extraction with phenol. The DNA content of the wound tissue nuclei was therefore measured after the extraction of RNA by phenol.

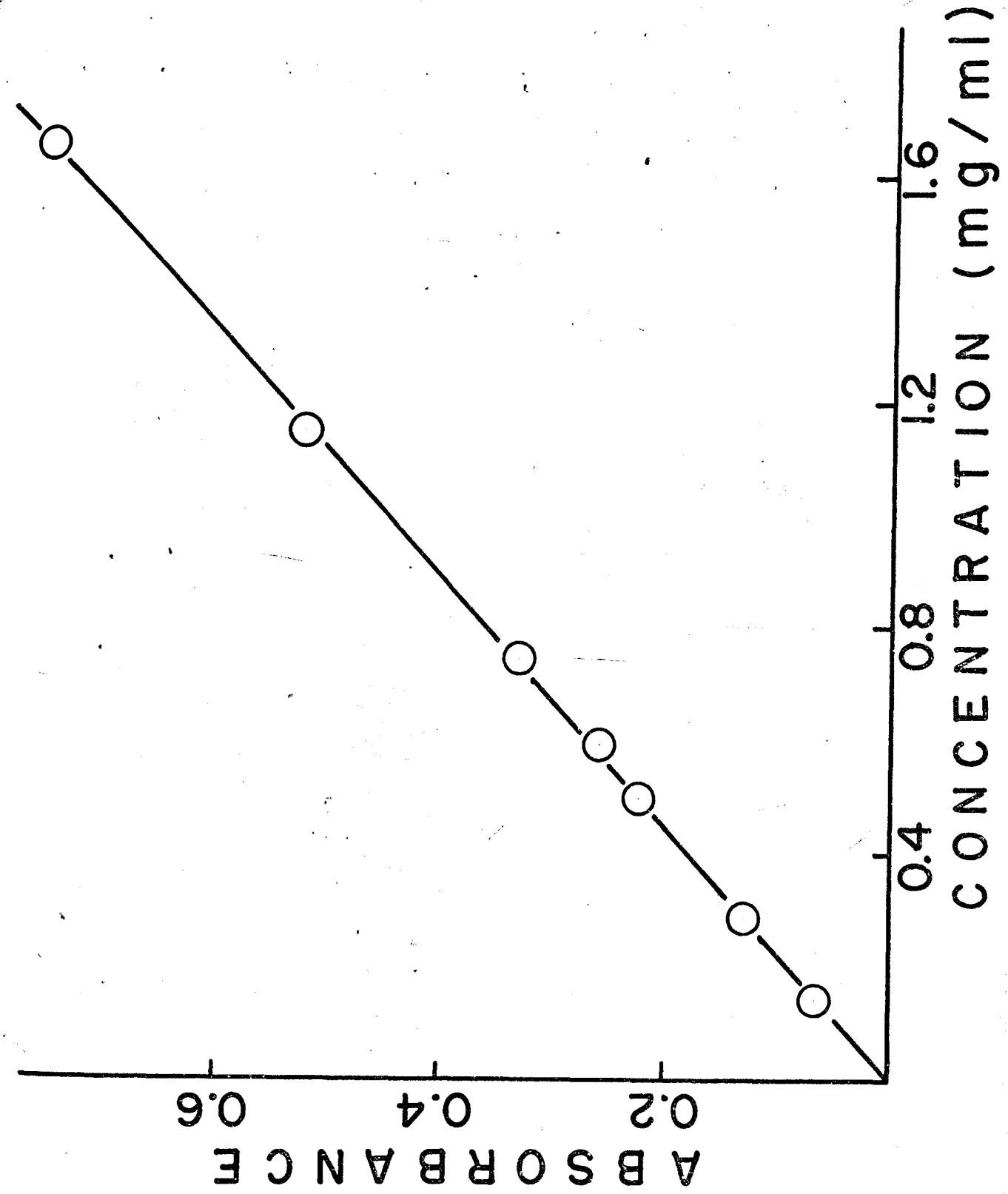
TABLE III

EFFECT OF PHENOL EXTRACTION ON DNA CONCENTRATION
IN LIVER NUCLEI AS DETERMINED BY DIPHENYLAMINE METHOD

Treatment of Nuclei	DNA Recovered from Suspension of Nuclei (mg./3.0 ml. Aliquot)*
untreated	76.4
phenol extracted	76.4

*Average of data obtained from analysis of three groups of tissue.

Fig. 3: Standard curve of DNA, expressed as absorbance at 600 nm vs. concentration as determined by the Diphenylamine method.



Liquid Scintillation Counting of UMP:

The UMP nucleotide in 1.0 ml. of 0.05 N HCl from the base composition determination step was added to 10 ml. of counting fluor containing 5 gms. of PPO and 100 gms. of naphthalene per liter of solution in dioxane. The counting sample was contained in a low potassium vial. After 30 minutes of dark adaptation, the samples were counted in a Beckman LS-250 liquid scintillation counter equipped with automatic quench correction and with a tritium Iso-set. Since the amount of nucleotide and hence, the activity, dealt with in this case was rather low, each sample was counted for 50.0 minutes, and at a counting error of 3%. After subtracting background activity, the specific activity of each sample was computed in terms of counts per minute per μM UMP. Specific activities of each fraction were then plotted against time in minutes after the subcutaneous injection of 5-³H-Uridine.

Each RNA fraction was then studied on the basis of its G+C/A+U ratio and its rate of turnover. For identification of possible matching fractions, seemingly similar curves were superimposed on each other and studied in conjunction with their G+C/A+U ratios.

RIBONUCLEASE IN REGENERATING WOUND TISSUE

To study the level of ribonuclease activity in regenerating wound tissue, and hence its effect on RNA metabolism during tissue regeneration, adult virgin female Sprague-Dawley rats were wounded and kept on protein-free diet as described earlier. On the 5th, 8th and 12th days after wounding, the regenerated wound tissue from groups of 10 rats each were harvested, immediately frozen in dry-ice, and kept under air-tight condition as mentioned in the RNA metabolism experiment.

Extraction of Ribonuclease:

Pooled frozen wound tissue (about 6 grams) from 3 to 5 rats were weighed, and then minced with a pair of scissors. The tissue sample was then transferred into a 16x150 mm glass homogenizer and 6 ml. of cold 0.2 M Tris buffer at pH 7.8 (the optimal pH of ribonuclease) was added. With the pestle of the homogenizer attached to a drill press, the wound tissue sample was homogenized for 18 minutes. The prolonged period of homogenization was necessary in order to bring about the breakage of the cells and the cell nuclei. The tissue homogenate was then centrifuged at 1,500 g for 45 minutes, in a refrigerated centrifuge. The supernatant containing the ribonuclease was carefully decanted into a 10 ml. graduated cylinder. Total volume of the enzyme supernatant was recorded. The whole operation was carried out at about 4°C. The amount of DNA in

the sediment at the bottom of the centrifuge tube was determined colorimetrically by the procedure and reaction with diphenylamine as described above.

Assay of Ribonuclease Activity:

To compare the level of activity of ribonuclease in the regenerating wound tissue samples, care should be taken to ensure that the amount of enzyme in the aliquot of supernatant used for the assay is the only limiting factor involved. In our study, 0.10, 0.20 and 0.30 ml. aliquots of the supernatant obtained from homogenized regenerating wound tissue were taken for the assay. A 1% yeast RNA in 0.2 M Tris buffer at pH 7.8 was used as the substrate (253, 254, 255). About 1.0 gm. of Torula Yeast RNA (Grade VI, Sigma Chemical Co., St. Louis, Missouri) was weighed, and then dissolved in 2.0 ml. of 1.0 M Tris solution. The pH of the solution was adjusted to a value of 7.8. Sufficient deionized water was then added to bring the total volume to 10.0 ml. Only substrates freshly prepared just prior to the assay were used.

Assay of Active And Bound Ribonuclease:

In a final volume of 0.5 ml., the incubation mixture contained 0.2 ml. of the 1% yeast RNA substrate, 0.2 M Tris buffer (pH 7.8), and aliquots of the enzyme supernatant (210, 255). The RNA substrate was added last, and the whole mixture was incubated at 37°C. After incubating for 20 minutes, the

enzyme reaction was stopped and the undigested RNA substrate was precipitated by the addition of 0.5 ml. of 1 N HCl in 76% ethanol. The mixture was thoroughly shaken and allowed to stand in an ice-bath for 30 minutes. The digested RNA in the mixture was separated by centrifugation at 4°C. 1.0 ml. of the clear supernatant was added to 30 ml. of water in a test tube, mixed well, and the absorbance at 260 nm determined. The assay was run in triplicate. For blanks, one assay tube contained only the substrate and Tris buffer, while another one contained an aliquot of the enzyme supernatant and Tris buffer but no RNA substrate. Optical density of each enzyme assay was subtracted by the combined absorbance of the two blanks. The net optical density was taken as a measure of the relative level of enzyme in the regenerating wound tissue. The above procedure measures the level of active ribonuclease.

To measure the relative level of total (ie. active + latent) ribonuclease in the wound tissue, we used 0.2 M Tris buffer at pH 7.8 containing 0.0025 M lead acetate in the place of lead-free Tris buffer in the incubation mixture as described above (210, 253, 254, 255, 256). The Pb^{++} inactivates the ribonuclease-inhibiting-proteins, therefore releasing the bound ribonuclease. Total ribonuclease activity in the Pb^{++} treated supernatant was then assayed as in the case of the active enzyme (21, 210, 223). By subtracting the level of active

enzyme from that of the total enzyme activity, the level of bound ribonuclease in the tissue was obtained. The above experiment was repeated three times with different groups of pooled wound tissue.

DNA DEPENDENT RNA POLYMERASE

To study the effect of DNA dependent RNA polymerase on RNA metabolism during tissue regeneration, an attempt was made to measure the level of RNA polymerase in regenerating wound tissue. Adult virgin female Sprague-Dawley rats were wounded and maintained on a protein-free diet as described above in the RNA metabolism experiment. On the 5th, 8th and 12th days after wounding, the regenerating wound tissue in groups of 20 rats each, was harvested and stored as described above. Here again, as in all the studies reported in this dissertation, all the rats were wounded, and the tissue samples were harvested, at about the same time of the day to minimize and standardize any variation due to diurnal rhythm.

Pooled frozen wound tissue from 7 rats were weighed. The nuclei were then isolated from the tissue samples by the processes of pulverization, homogenization and sucrose gradient centrifugation as described above. About 6 ml. of 0.05 M Tris buffer at pH 7.9 (optimum pH of DNA dependent RNA polymerase) was added to the isolated nuclei. The suspension was then homogenized for 2-3 minutes in order to rupture the nuclei in it. The amount of RNA polymerase in aliquots of this chromatin suspension was assayed as described below (131, 137). In another aliquot of the chromatin suspension, the amount of DNA in it was determined by the diphenylamine reaction method as

described in detail previously.

Template activity of DNA had been shown to vary with time, for instance, in the regenerating liver (8, 43, 253). Different areas of the DNA are exposed for transcription, depending on the metabolic needs of the organism at various moments (38, 81, 236). Hence, the base composition of the RNA synthesized, as dictated by the area of the DNA exposed for transcription, may also vary, for instance, at different stages of tissue regeneration (19, 24, 42, 144, 161). In our assay of the level of RNA polymerase in regenerating wound tissue, we therefore use pooled and stripped DNA as template. This also ensured that there were more gene sites than there was RNA polymerase available in the assay; hence the level of RNA polymerase was the only limiting factor involved.

To prepare the stripped DNA template, pooled liver from 5-6 rats was homogenized in 0.25 M sucrose solution, buffered at pH 7.9, and the nuclei were separated by centrifugation as described before. The isolated nuclei were then suspended in 20 volumes of 0.20 N HCl with gentle stirring, at 10°C. After 2 hours, the suspension was centrifuged at 700 g for 45 minutes. The supernatant was then discarded. The precipitate obtained was washed once with 0.05 M Tris buffer, pH 7.9, and recentrifuged (9, 246). The resultant precipitate which contained the stripped DNA was then suspended in 5 volumes of the same Tris

buffer. Aliquots of this stripped DNA preparation were used as template for the assay of RNA polymerase.

Assay of RNA Polymerase:

To assay the level of RNA polymerase in regenerating wound tissue the reaction mixture contained 0.2 ml. of the substrate, an aliquot of the chromatin suspension (0.10 ml., 0.20 ml., and 0.30 ml. respectively), 0.5 ml. of the stripped DNA template suspension and sufficient amount of 0.05 M Tris buffer at pH 7.9 to bring the final volume of the mixture to 1.0 ml. (131, 137). Each 0.2 ml. of the substrate contained 5.0 μ M MgCl_2 , 1.0 μ M MnCl_2 , 12 μ M 2-Mercaptoethanol (Sigma Chemical Co., St. Louis, Missouri), 0.2 μ M each of GTP, CTP and ATP (Sigma Chemical Co.), and 4 μ Ci (in 0.24 μ M) of 5-³H-UTP (Schwarz BioResearch, Orangeburg, New York), in 0.05 M Tris buffer at pH 7.9 (14, 232). In each case, the substrate was added last. The reaction mixture was then incubated at 37° for 10.0 minutes. Following that, the reaction was stopped and the transcribed RNA precipitated by the addition of 1.0 ml. of 1.0 N HCl in 76% ethanol (34, 40, 109, 243). After chilling in an ice-bath for 30 minutes, it was centrifuged at 1,500 g for 45 minutes. The precipitate obtained was washed twice with 2.0 ml. of the same ice-chilled acid ethanol, and re-centrifuged. The resultant precipitate was dried and put in a liquid scintillation vial. Twelve ml. of a liquid scintillation fluor containing 6 gm. of PPO in 1.0 l.

of toluene and 200 ml. of Bio-Solv (BBS 3, Beckman Instrument Co., Lincolnwood, Illinois) was then added. The samples were counted in a Beckman LS-250 liquid scintillation counter. The specific activity of each sample was taken as a relative measure of the amount of RNA polymerase.

HISTONES AND GENE DEREPRESSION

To study the mechanism of gene derepression involved in regenerating wound tissue, the rats were wounded as described previously. On the 5th, 8th and 12th days after wounding, the wounded animals, in groups of 12 rats each, were injected subcutaneously with 1.0 ml. of solution containing 50 μ Ci of L-(methyl- 3 H)-Methionine and 1.25 mCi of $^{32}\text{PO}_4^{=}$ (Schwarz Bio-Research, Orangeburg, New York), per rat. After 60 minutes, the regenerated tissue was harvested and stored as described before. In an identical batch of wounded rats and on the same days as above, each of 12 rats per group was injected subcutaneously with 1.0 ml. of solution containing 1.25 mCi of $\text{C}^3\text{H}_3-\overset{\text{O}}{\underset{\text{||}}{\text{C}}}-\text{O}^-$ (Amersham-Searle BioResearch, Arlington Heights, Ill.). After 120 minutes, the regenerated tissue was harvested and stored as before. Each group of tissue was then processed in the same manner as described below. Owing to the short half-life of phosphorus-32, the tissue samples containing radioactive phosphate were processed immediately after harvesting.

Extraction of Histones:

Nuclei were isolated from pooled frozen tissue (6 rats) by homogenization and sucrose gradient centrifugation as described in the RNA metabolism experiment. To extract the lysine-rich histones in the nuclei, the isolated nuclei were suspended in 20 volumes of 0.1 M citric acid for 45 minutes, in the cold, with

gentle stirring, as described by Allfrey et al. (9). The whole suspension was then centrifuged at 600 g for 45 minutes.

The lysine-rich histones extracted in the supernatant was carefully siphoned off and precipitated with 10 volumes of acetone (18, 2, 36, 246).

The nuclear residue was then suspended in 20 volumes of 0.2 N HCl for 60 minutes, in the cold, with gentle stirring. It was then centrifuged at 600 g. The arginine-rich histone in the supernatant was carefully pipetted off and precipitated with 10 volumes of acetone (8, 246). The amount of DNA in the nuclear residue was then determined by reaction with diphenylamine as described above (279A; 282, 283).

After standing in the cold room over-night, the histone precipitates were centrifuged down at 1,500 g. About 3 ml. of deionized water was added to each histone precipitate, followed by freeze-drying.

To separate the histones from any other protein which might be present in the extract, the histone fractions were purified by Bio-Gel filtration. Each histone fraction was dissolved in 0.05 M Tris buffer, pH 7.2, and then loaded onto a 30.0 x 1.0 cm. Bio-Gel P 30 (200 to 400 mesh) column. The Bio-Gel P 30 has a molecular exclusion of 30,000, which would allow histones (M.W. = 20,000) to pass through, and exclude larger proteins like collagen. The histones were eluted with

0.05 M Tris buffer, pH 7.2. The whole elution process was monitored at 280 nm with a Cary 15 spectrophotometer. Each purified histone fraction was recovered by precipitation with acetone as described before.

To verify that each fraction actually contained histone, an aliquot of each purified histone extract was dissolved in water. A volume of 1.0 N NH_4OH was then added to each solution. After shaking and standing for a few minutes, a precipitate was formed in each tube, indicating the presence of histone in it.

To assess the specific activity of each type of isotope in the lysine-rich and the arginine-rich histones, each histone fraction extracted from regenerating wound tissue was dissolved in 15.0 ml. of counting fluor. The counting fluor contained 6 gm. of PPO (Sigma Chemical Co., St. Louis, Missouri) in 1.0 l. of toluene (279A, 283) and 200 ml. of Biosolv (BBS3, Beckman, Lincolnwood, Illinois), per 1,200 ml. solution. Each sample was then counted on a Beckman LS-250 liquid scintillation counter.

Chapter V

RESULTS AND DISCUSSION

NUCLEAR RNA METABOLISM IN REGENERATING TISSUE

As mentioned earlier, present day concepts of cellular biology emphasize the role of nucleic acids in directing cellular activities and functions. The necessary information for metabolic processes is contained in the nucleotide sequences of DNA. This information is carried to the sites of protein synthesis by the complementary RNA. In an attempt to unravel some of the molecular mechanisms regulating tissue regeneration, such as the rate of protein synthesis at different stages of wound healing, we have conducted a study of the metabolism of nuclear RNA of regenerating cutaneous tissue.

Table IV shows the level of RNA extracted from nuclei of regenerating cutaneous tissue at various times after wounding. The amount of nuclear RNA in each extract varies as tissue regeneration progresses. The level of total nuclear RNA per cell appears to increase rapidly to a maximal level in about 8 days and then begins to decline, hence paralleling the relative rates of both cellular protein and collagen synthesis in this tissue as reported by Williamson et al., (134, 279A, 282, 283). Table IV also shows a comparison of the amount of RNA in various cell compartments. It can be seen that the changes in RNA content as tissue regeneration proceeds are due largely to

TABLE IV

DISTRIBUTION OF RNA EXTRACTED FROM NUCLEI IN REGENERATING WOUND TISSUE

Extract		Days after wounding ^a					
		5		8		12	
No.	pH	$\frac{\mu\text{M RNA}}{\text{mg. DNA}}$	%	$\frac{\mu\text{M RNA}}{\text{mg DNA}}$	%	$\frac{\mu\text{M RNA}}{\text{mg DNA}}$	%
1	7.2	91.2 ± 3.9^c	60	190.0 ± 8.5^c	61	111.8 ± 11.4	55
2	7.2	22.5 ± 2.7	15	46.8 ± 5.6	15	41.4 ± 4.4	19
3-4	7.2	20.9 ± 2.3	14	26.8 ± 2.8	9	25.5 ± 2.1	12
5	8.6	7.1 ± 2.0	5	23.2 ± 2.5	8	13.8 ± 1.5	6
6-7	8.6	9.3 ± 2.1	6	22.1 ± 1.4	7	18.6 ± 2.3	9
Nuclear RNA		151.0		308.9		211.1	
Total RNA ^b		540		720		630	
Cytoplasmic RNA		389		411		419	

^aData for each experimental day was obtained from the analysis of 4 pools of 20 samples of regenerating tissue.

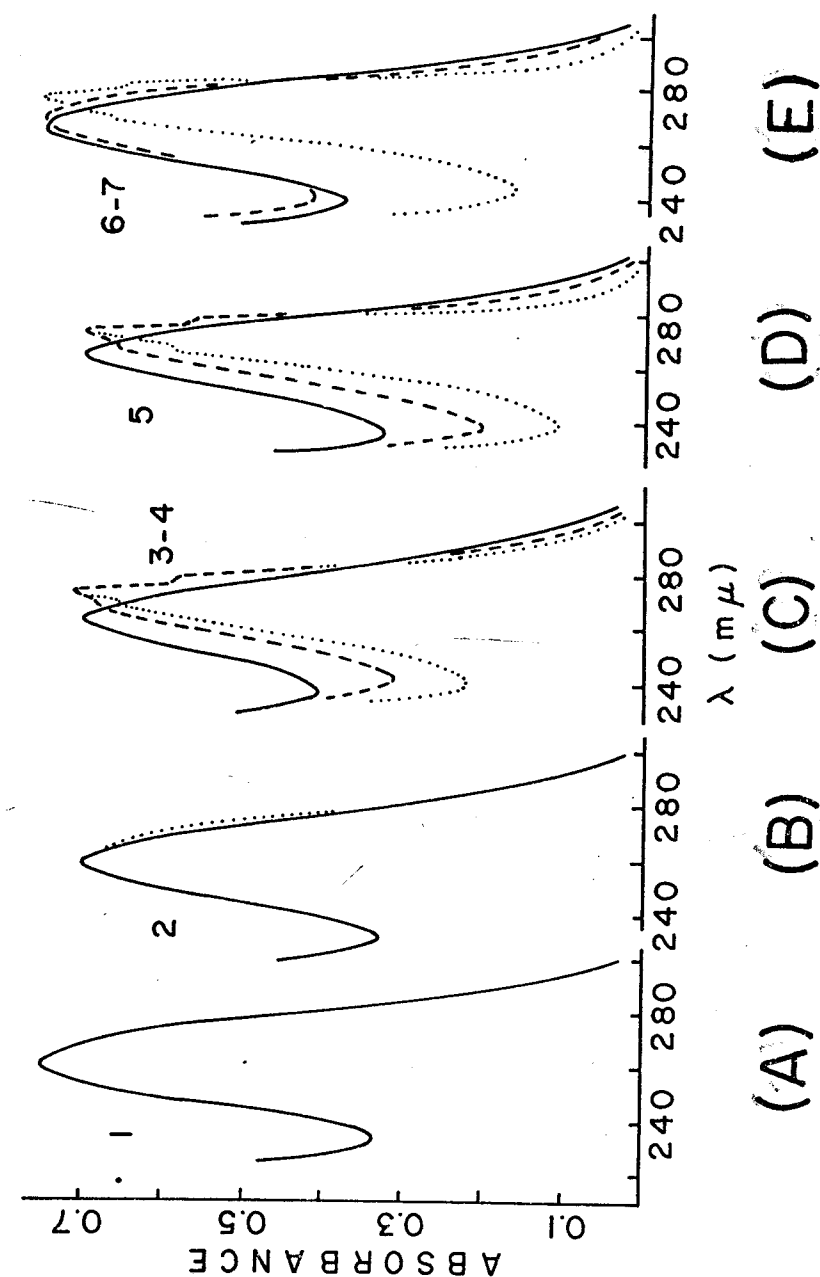
^bCalculated from data previously reported from this laboratory (273-275)

^cMean \pm standard deviation.

changes in the amount of RNA in the nuclei. It appears from our data, that the variation in the amount of RNA in the regenerating tissue is primarily due to changes in the amount of RNA per cell, rather than due to fluctuations in the relative number of cells in the tissue as reported by earlier studies (276).

To find out whether there are qualitative changes in the nuclear RNA as wound healing progresses, a preliminary indication of the RNA populations was obtained from the absorption spectra of the various RNA Extracts (Fig. 4). The spectra of Extracts 1 (Fig. 4a), representing some 50-60% of the total nuclear RNA, suggest that most of the RNA moieties are unchanged during the period of regeneration studied. The similarity of the spectra of the first and second neutral pH extracts points to the possibility that much of the RNA in both may be similar. Again, the spectra of RNA in the second extract (Fig. 4b) are practically unchanged as tissue regeneration progresses. These data suggest that during the course of wound tissue formation most of the RNA in the nuclei remains the same. A small difference in the spectrum of the 12th day 2nd extract, however, is noticeable from those of the 5th and 8th days (Fig. 4b), suggesting the presence of an appreciable amount of a different type of RNA in the 12th day extract. The spectra of the RNA in the other extracts (Fig. 4c, 4d, 4e), representing some

Fig. 4: Absorption spectra of the RNA extracted from the nuclei of regenerating cutaneous tissue at different stages of regeneration. (—) the spectrum of the nuclear RNA obtained 5 days after wounding; (— —), 8 days; (...), 12 days. (a) Extract 1, first neutral pH extract; (b) Extract 2, second neutral extract; (c) Extract 3-4, combined third and fourth neutral extract; (d) Extract 5, first extract at pH 8.6; (e) Extract 6-7, combined second and third extracts at pH 8.6. In (a) and (b) the three spectra are so similar that only a single curve is apparent.



25% of the total, indicate a great change in the base composition of the RNA population. The shift of the absorption maxima of these spectra to longer wavelengths at neutral pH implies the appearance of RNA with a relatively decreased proportion of cytidylic and guanylic acid residues as tissue regeneration proceeds.

To verify these qualitative changes in the RNA population of the nuclei, each RNA extract was fractionated with an anion exchange ECTEOLA cellulose column. Nucleotide analysis of each RNA fraction was carried out by mild alkaline hydrolysis, followed by separation with a Dowex 50W-X4 cation exchange column. Table V shows the G+C/A+U ratio of each RNA fraction. RNA moieties in fraction 1A appear to change as regeneration progresses, judging from the G+C/A+U ratios (Table V) and the rates of synthesis and turnover (Fig. 5, 6, 7). Although the type of nuclear RNA as discussed earlier varies greatly, the relative abundance of this RNA fraction remains fairly constant (Table VI). All the RNA, however, remain as the low G+C/A+U types. The RNA molecules in fraction 1B change in terms of G+C/A+U ratio (Table V), the rates of synthesis and turnover (Fig. 5, 6, 7), and the amount and percentage of RNA (Table VI). Fractions 1C and 1D are composed mainly of high G+C/A+U types of RNA, and appear to be present throughout the experimental period (Table V and Fig. 5, 6, 7). The content and relative

TABLE V

BASE RATIO CHARACTERISTICS OF NUCLEAR RNA FRACTIONS OF
REGENERATING CUTANEOUS TISSUE

Sample #	G+C/A+U *		
	Days after wounding		
	5	8	12
1 A	0.13	0.34	0.40
1 B	0.99	0.89	1.12
1 C	1.33	1.38	1.50
1 D	1.55	1.73	1.67
2 A	0.20	0.24	0.40
2 B	0.76	0.71	0.74
2 C	1.35	1.27	1.10
2 D	1.59	1.35	0.83
3-4 A	0.27	0.31	0.41
3-4 B	0.33	0.36	0.48
3-4 C	1.06	1.17	0.49
3-4 D	1.68	0.35	0.42
5 A	0.18	0.31	0.21
5 B	0.36	0.34	0.42
5 C	1.04	0.58	0.83
5 D	1.26	0.37	0.25
6-7 A	0.19	0.26	0.27
6-7 B	0.68	0.40	0.43
6-7 C	1.31	0.67	0.36
6-7 D	1.41	0.29	0.37

* Data for each experimental day was obtained from the analysis of 4 pools of 20 samples of regenerating cutaneous tissue.

Fig. 5: The rate of formation and turnover of nuclear RNA measured in terms of counts per minute per mM UMP against time after administration of 118 μ C of 5- 3 H-Uridine. The curves represent the fractions of Extract 1 eluted from ECTEOLA column with LiCl. Extract 1 is the first extraction by Tris buffer, pH 7.2, of a phenol suspension of nuclear material from the wound tissue which has regenerated for 5 days. Fraction A is obtained by elution with 0.2 M LiCl; B, with 0.4 M LiCl; C, with 0.6 M LiCl in Tris buffer, pH 7.2; D, with 1.0 M LiCl in 0.05 M KOH. Each point represents the data from the analysis of 4 pools of 20 samples of regenerating tissue.

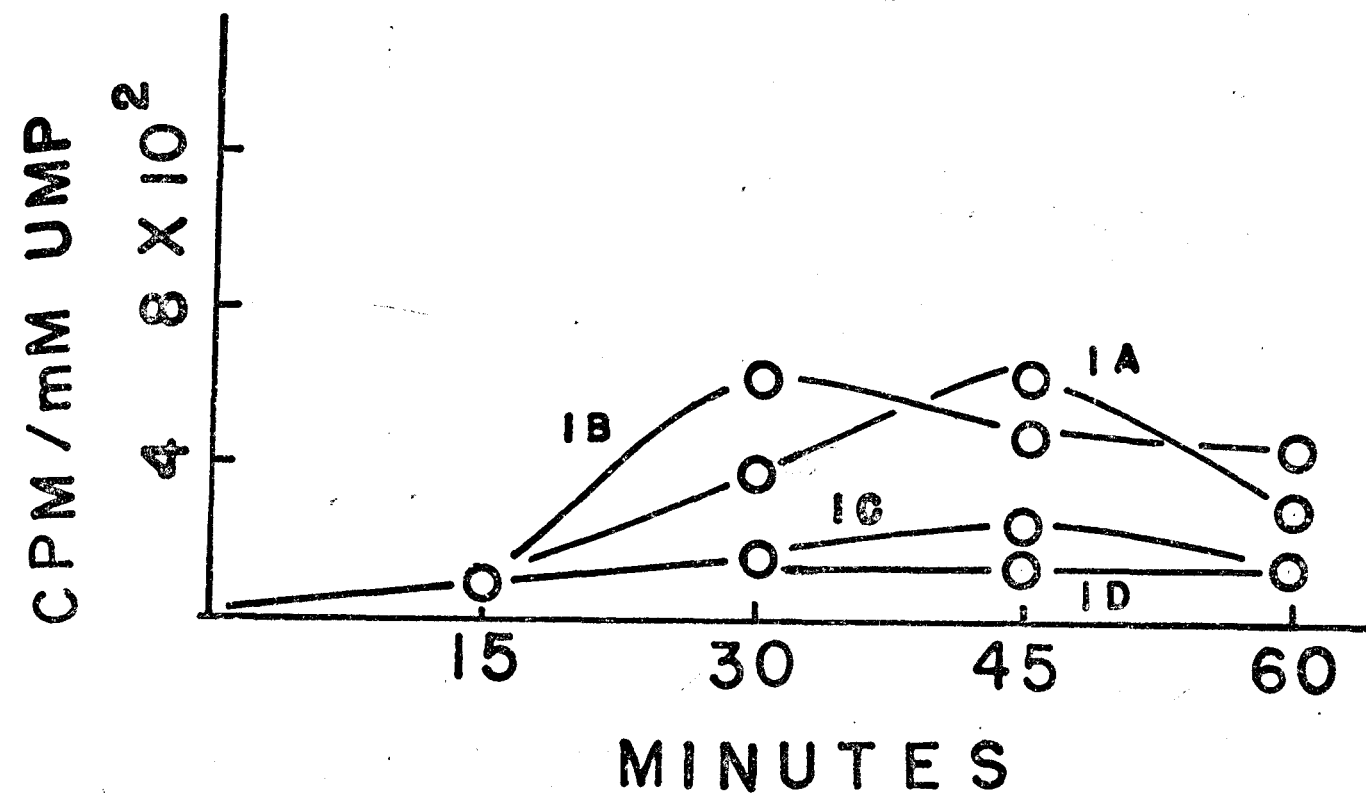


Fig. 6: The rate of formation and turnover of nuclear RNA fractions of Extract 1 of nuclei isolated from wound tissue which has regenerated for 8 days. Symbols as in Fig. 5.

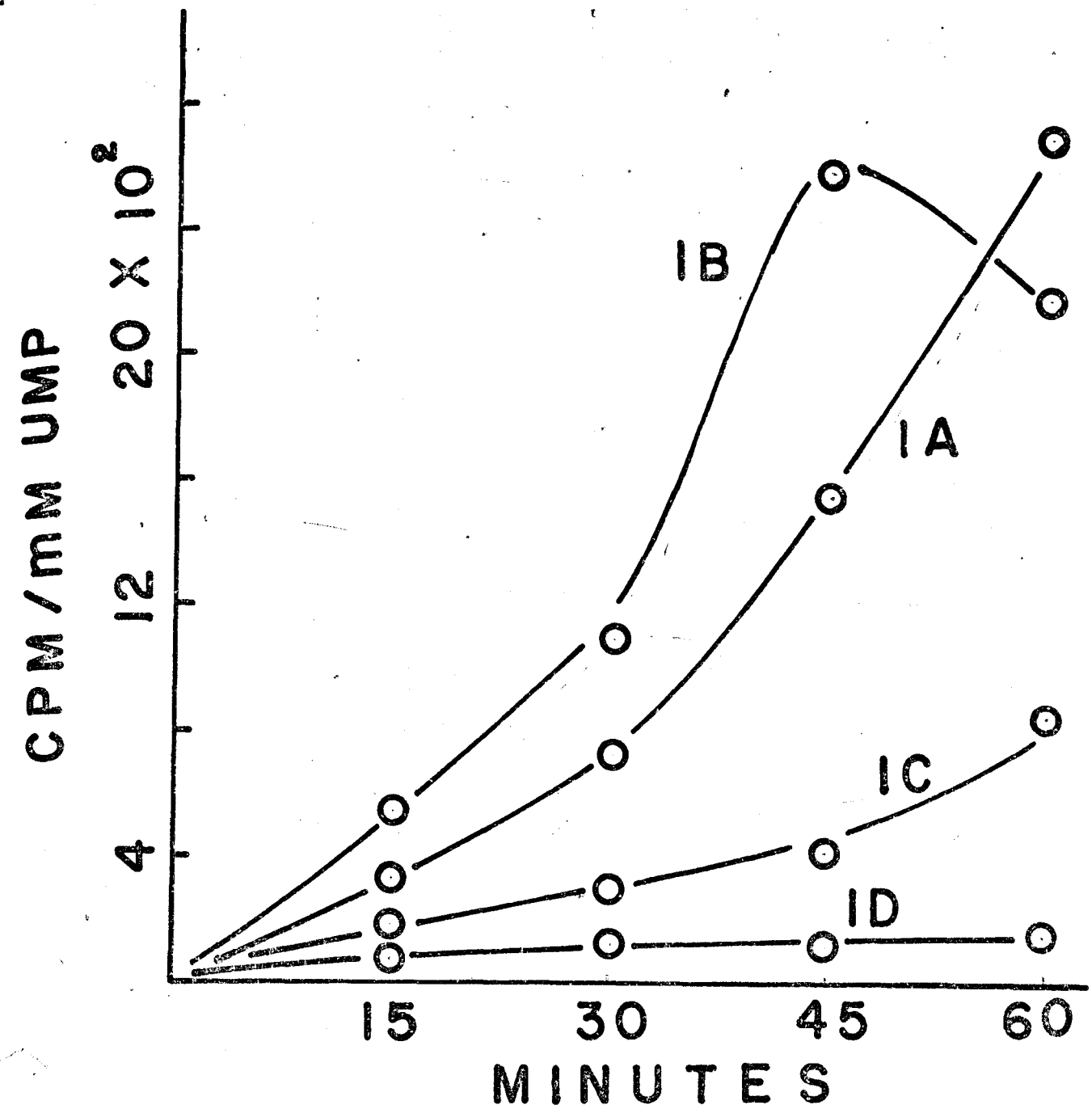


Fig. 7: The rate of formation and turnover of nuclear RNA fractions of Extract 1 of nuclei isolated from wound tissue which has regenerated for 12 days. Symbols as in Fig. 5.

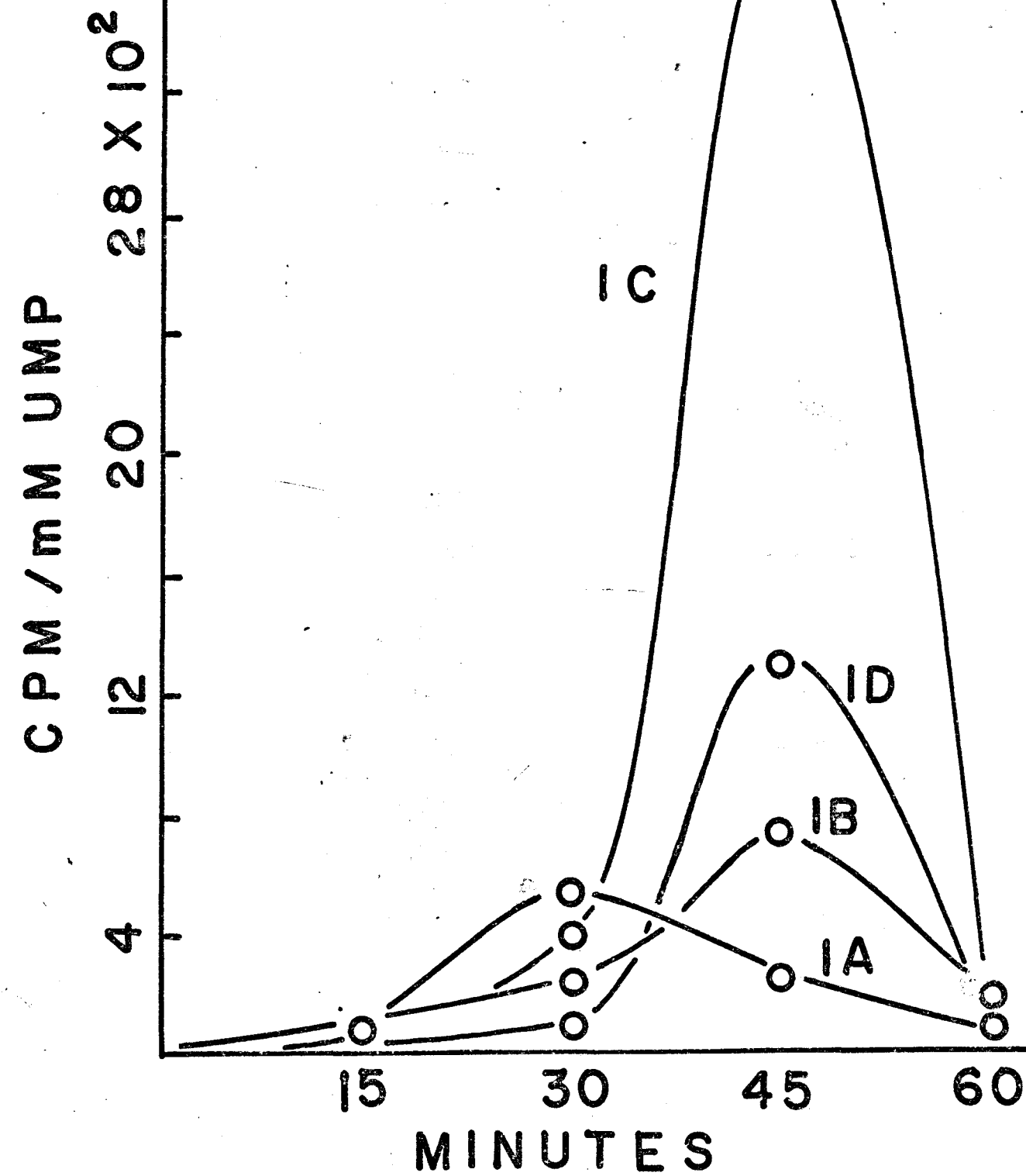


TABLE VI
RELATIVE ABUNDANCE OF NUCLEAR RNA FRACTIONS OF
REGENERATING CUTANEOUS TISSUE

Sample #	% yield of nucleotide in each RNA fraction*		
	Days after wounding		
	5	8	12
1 A	2.94	2.61	2.66
1 B	1.35	3.59	4.88
1 C	5.89	10.00	7.92
1 D	50.23	46.91	37.52
2 A	0.93	1.87	3.59
2 B	0.74	2.32	3.78
2 C	2.55	3.34	3.98
2 D	10.70	7.62	8.25
3-4 A	0.67	1.40	3.14
3-4 B	0.90	1.71	4.05
3-4 C	1.60	1.63	3.27
3-4 D	10.64	2.31	3.03
5 A	0.47	2.09	1.30
5 B	0.54	1.13	1.26
5 C	0.96	1.77	1.72
5 D	2.72	2.51	2.23
6-7 A	0.31	1.72	2.27
6-7 B	0.30	1.31	2.79
6-7 C	1.22	1.21	1.94
6-7 D	4.36	2.96	1.73

* Same footnote as in Table V.

proportion of this high G+C/A+U types of RNA vary considerably (Table VI), signifying perhaps the tremendous differences in metabolic and cellular activities at different stages of tissue regeneration. In agreement with the spectral data, nucleotide analysis shows that the majority of the RNA in Extract 1 remains unchanged during tissue regeneration.

RNA molecules in Extract 2 remain virtually the same on the 5th and 8th days after wounding, based on the G+C/A+U ratios (Table V) and the rates of synthesis and turnover of fractions 2A, 2B, 2C and 2D (Fig. 8, 9, 10). Fractions 2B and 2C remain essentially unchanged on the 12th day. A significant amount of variation in fractions 2A and 2D on the 12th day, however, is noticeable. The most metabolically dramatic change occurs in fraction 2D, which seemingly is replaced by RNA molecules having a much lower proportion of cytidylic and guanylic acid residues on the 12th day. As shown in Table VI, despite the similarity of most of the RNA fractions in Extract 2, the relative proportion of each type of RNA changes markedly from day to day, stressing perhaps the dynamic state of changes in the metabolic pattern of a regenerating tissue. Judging from the high base ratios (viz. $G+C/A+U = 1.4 \pm 0.4$), and the elution data, it appears that most of the RNA molecules in fractions 1C, 1D, 2C and 2D are ribosomal RNA (r-RNA), as reported by Georgiev et al. (82, 112A). This observation is in agreement

Fig. 8: The rate of formation and turnover of nuclear RNA fractions of Extract 2 of nuclei isolated from wound tissue which has regenerated for 5 days. Extract 2 was extracted at pH 7.2. Symbols as in Fig. 5.

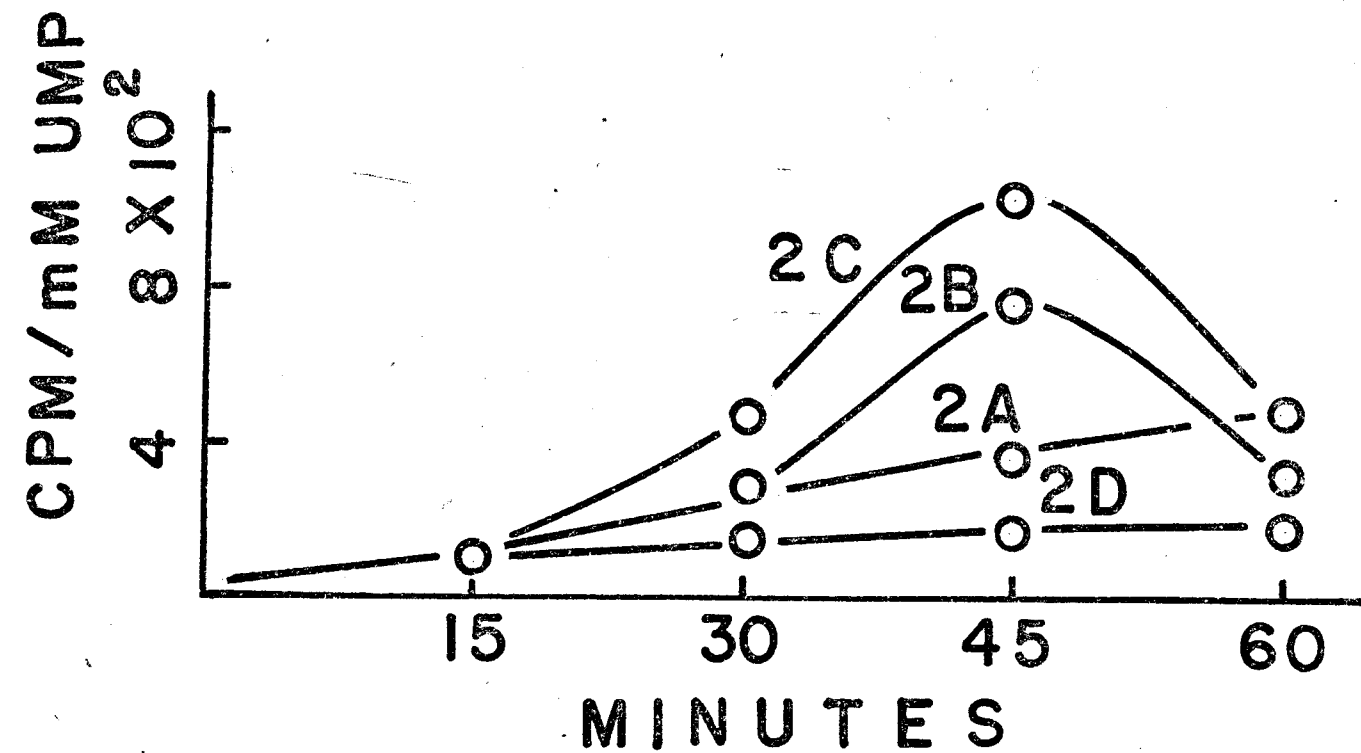


Fig. 9: The rate of formation and turnover of nuclear RNA fractions of Extract 2 of nuclei isolated from wound tissue which has regenerated for 8 days. Extract 2 was extracted at pH 7.2. Symbols as in Fig. 5.

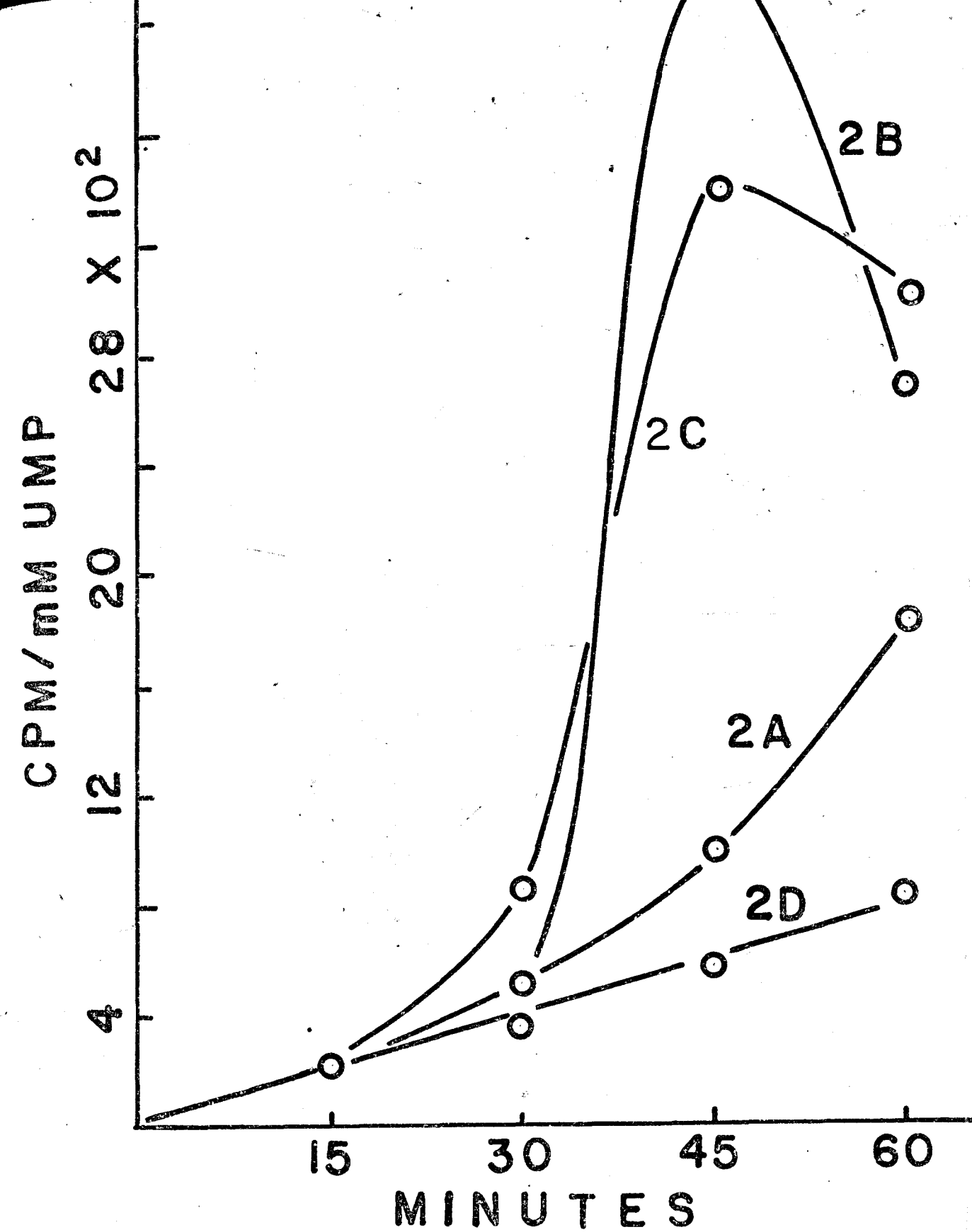
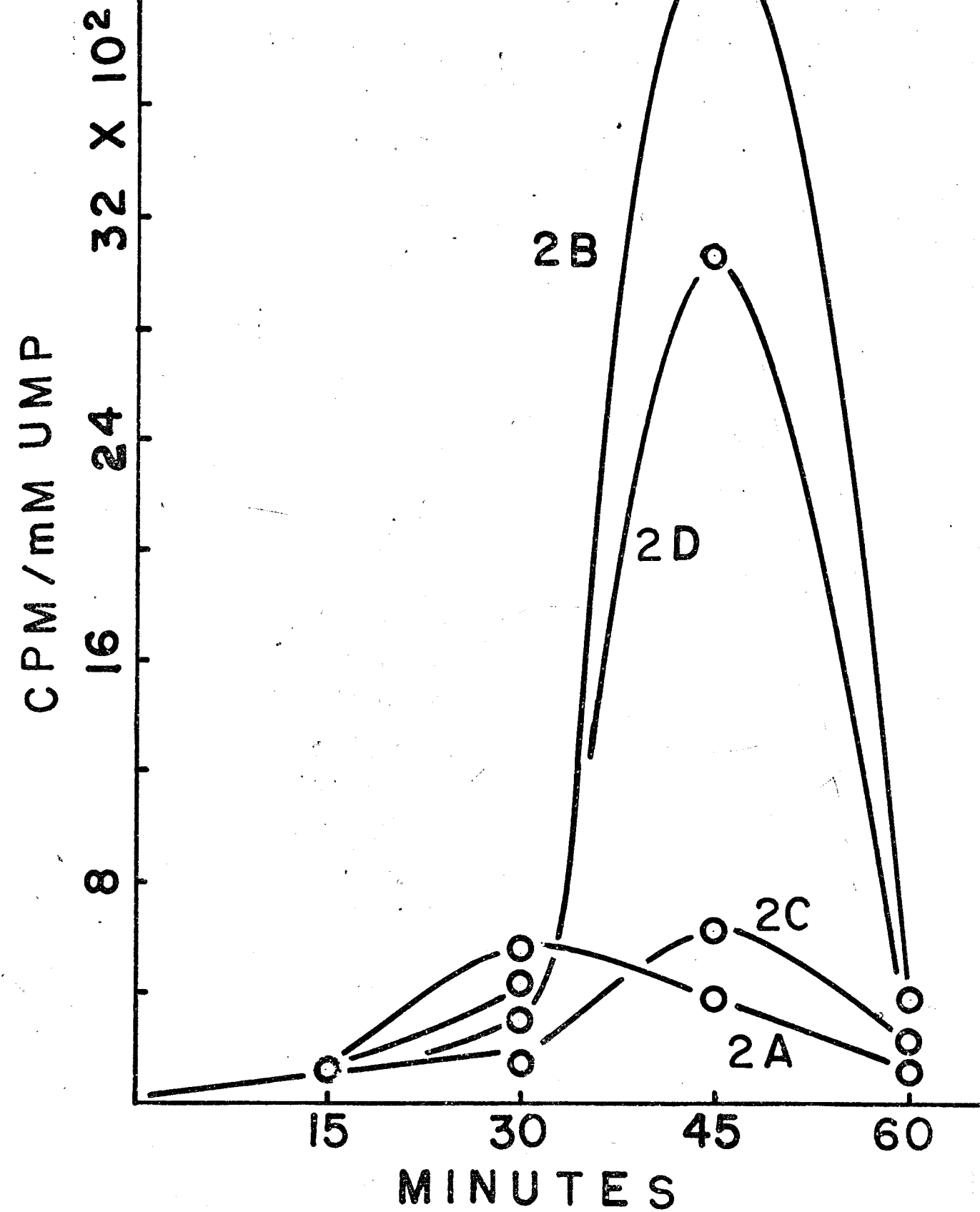


Fig. 10: The rate of formation and turnover of nuclear RNA fractions of Extract 2 of nuclei isolated from wound tissue which has regenerated for 12 days. Extract 2 was extracted at pH 7.2. Symbols as in Fig. 5.



with the sedimentation centrifugation data of similar RNA fractions from regenerating granulomas of sponge implant (4).

The RNA molecules in Extract 3-4 change radically during the experimental period (Tables V and VI, and Fig. 11, 12, 13). Fractions 3-4A and 3-4B are composed mainly of low G+C/A+U types of RNA, presumably DNA-like RNA. Fraction 3-4 C and 3-4D are comprised of relatively high G+C/A+U types of RNA at the beginning of the experimental period. However, as regeneration progresses the RNA populations change dramatically; the base ratios change from high to relatively low values, presumably as the result of drastic increase in the proportion of the DNA-like RNA. RNA in Extracts 5 and 6-7 appear to consist mainly of the DNA-like RNA with a relatively low G+C/A+U ratio (Table V). Despite the seemingly similar low base ratios, these RNA fractions are not necessarily the same in amount and type, as indicated by the data in Table VI and Figures 14, 15, 16, 17, 18 and 19. As in extract 3-4, the most obvious and pervading picture in extracts 5 and 6-7 is the replacement of the high G+C/A+U RNA by the low base ratio DNA-like RNA molecules as collagen and cellular protein synthesis and deposition commences in the wound tissue.

Judging from the spectra and the nucleotide analysis data, nuclear RNA metabolism in the wound tissue seems to entail a steady increase in the amount and types of DNA-like RNA having

Fig. 11: The rate of formation and turnover of nuclear RNA fractions of Extracts 3-4 of nuclei isolated from wound tissue which has regenerated for 5 days. Extracts 3-4 were extracted at pH 7.2. Symbols as in Fig. 5.

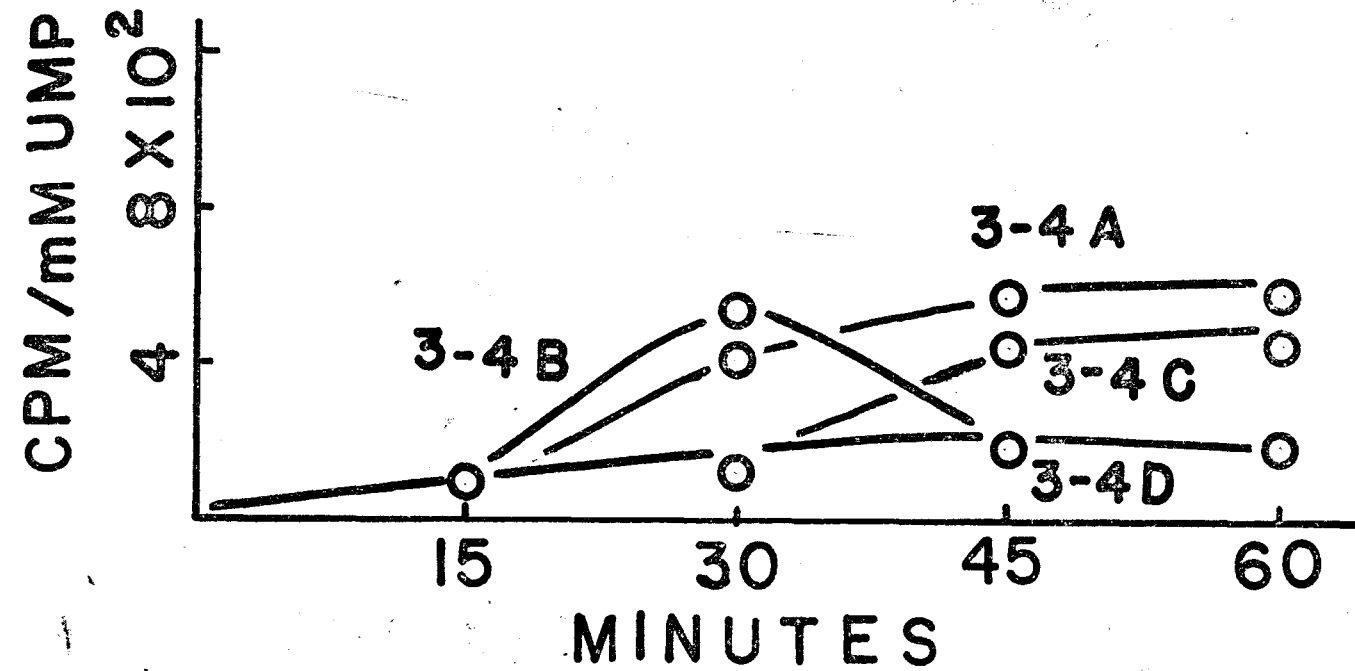


Fig. 12: The rate of formation and turnover of nuclear RNA fractions of Extracts 3-4 of nuclei isolated from wound tissue which has regenerated for 8 days. Extracts 3-4 were extracted at pH 7.2. Symbols as in Fig. 5.

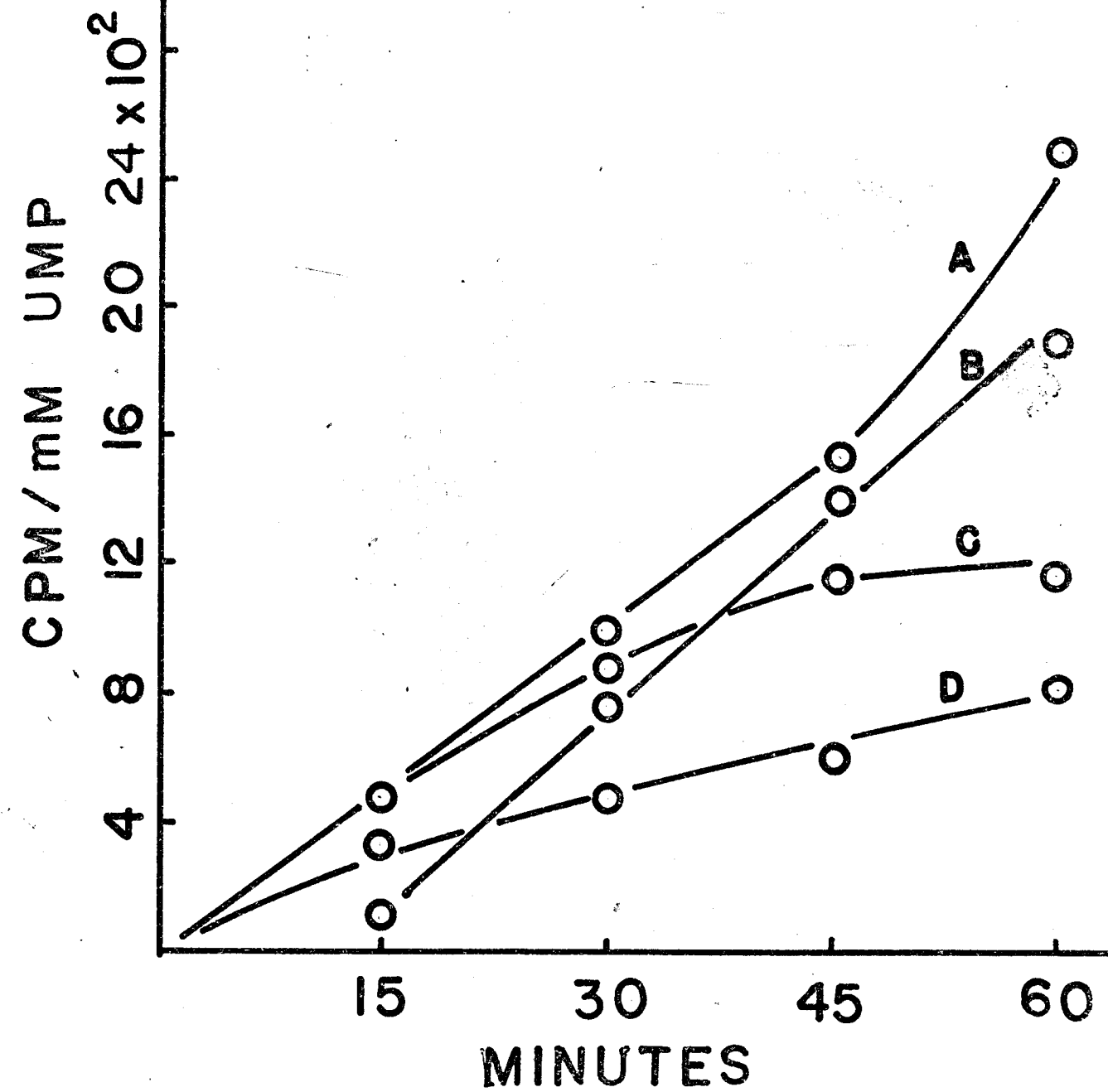


Fig. 13: The rate of formation and turnover of nuclear RNA fractions of Extracts 3-4 of nuclei isolated from wound tissue which has regenerated for 12 days. Extracts 3-4 were extracted at pH 7.2. Symbols as in Fig. 5.

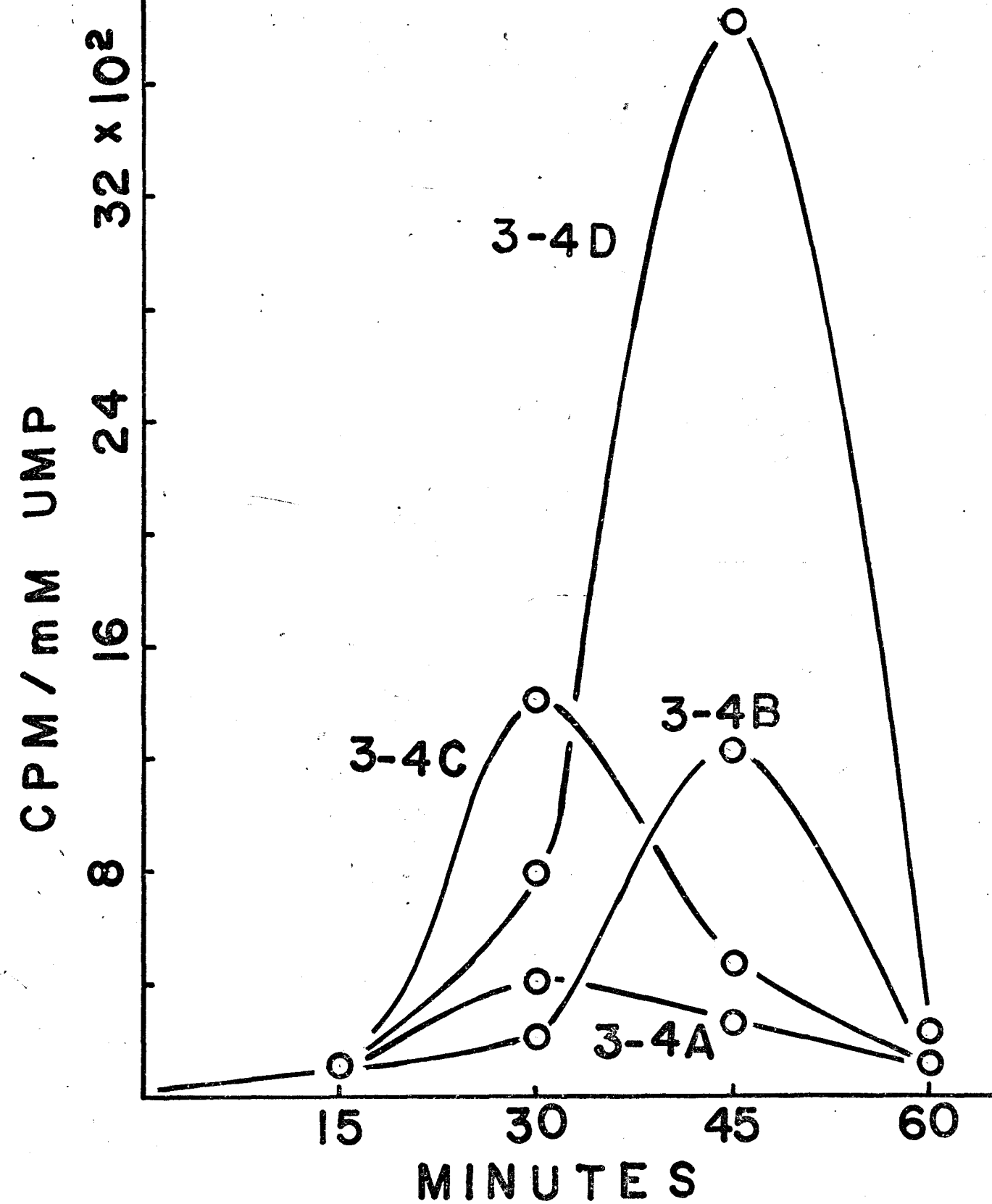


Fig. 14: The rate of formation and turnover of nuclear RNA fractions of Extract 5 of nuclei isolated from wound tissue which has regenerated for 5 days. Extract 5 were extracted at pH 8.6. Symbols as in Fig. 5.

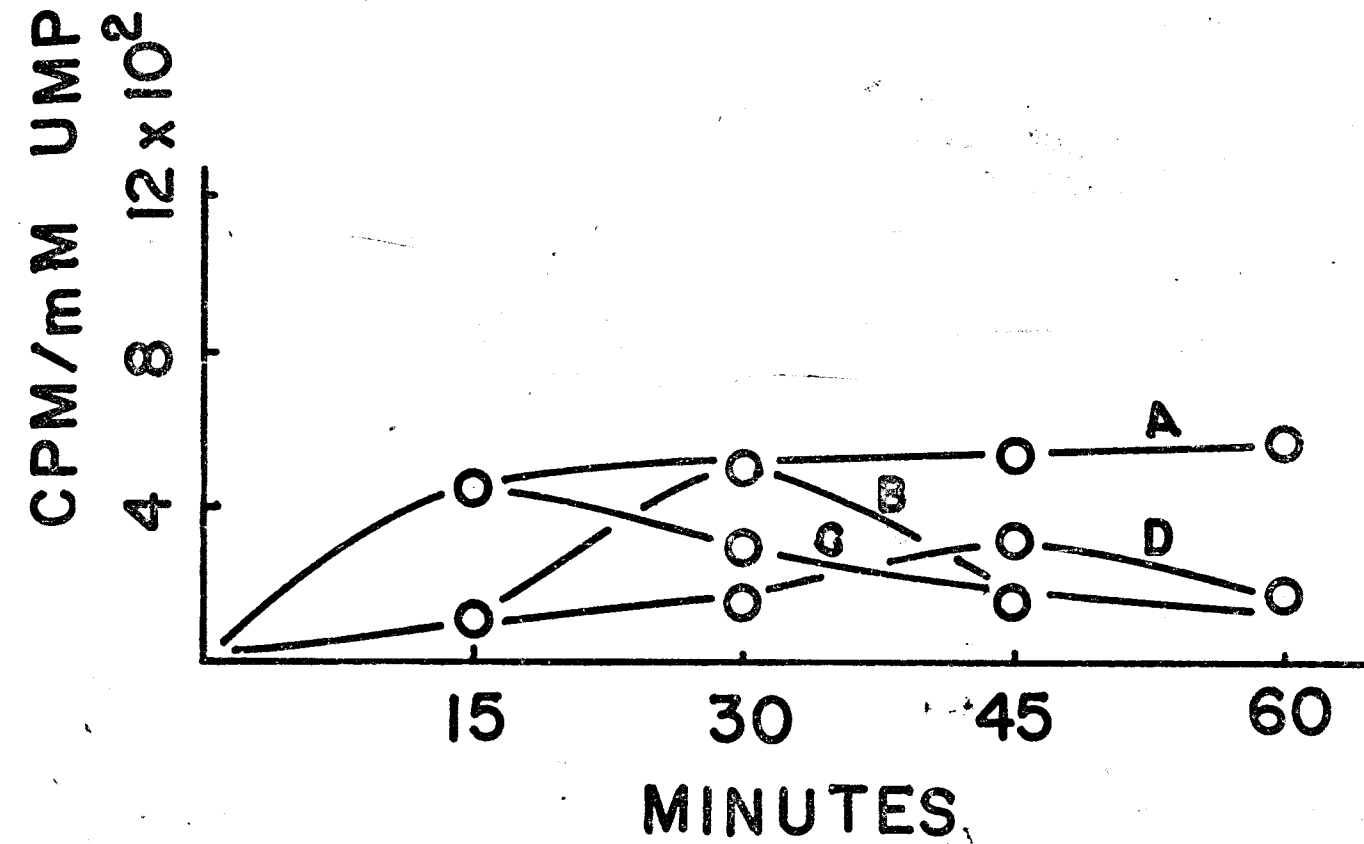


Fig. 15: The rate of formation and turnover of nuclear RNA fractions of Extract 5 of nuclei isolated from wound tissue which has regenerated for 8 days. Extract 5 were extracted at pH 8.6. Symbols as in Fig. 5.

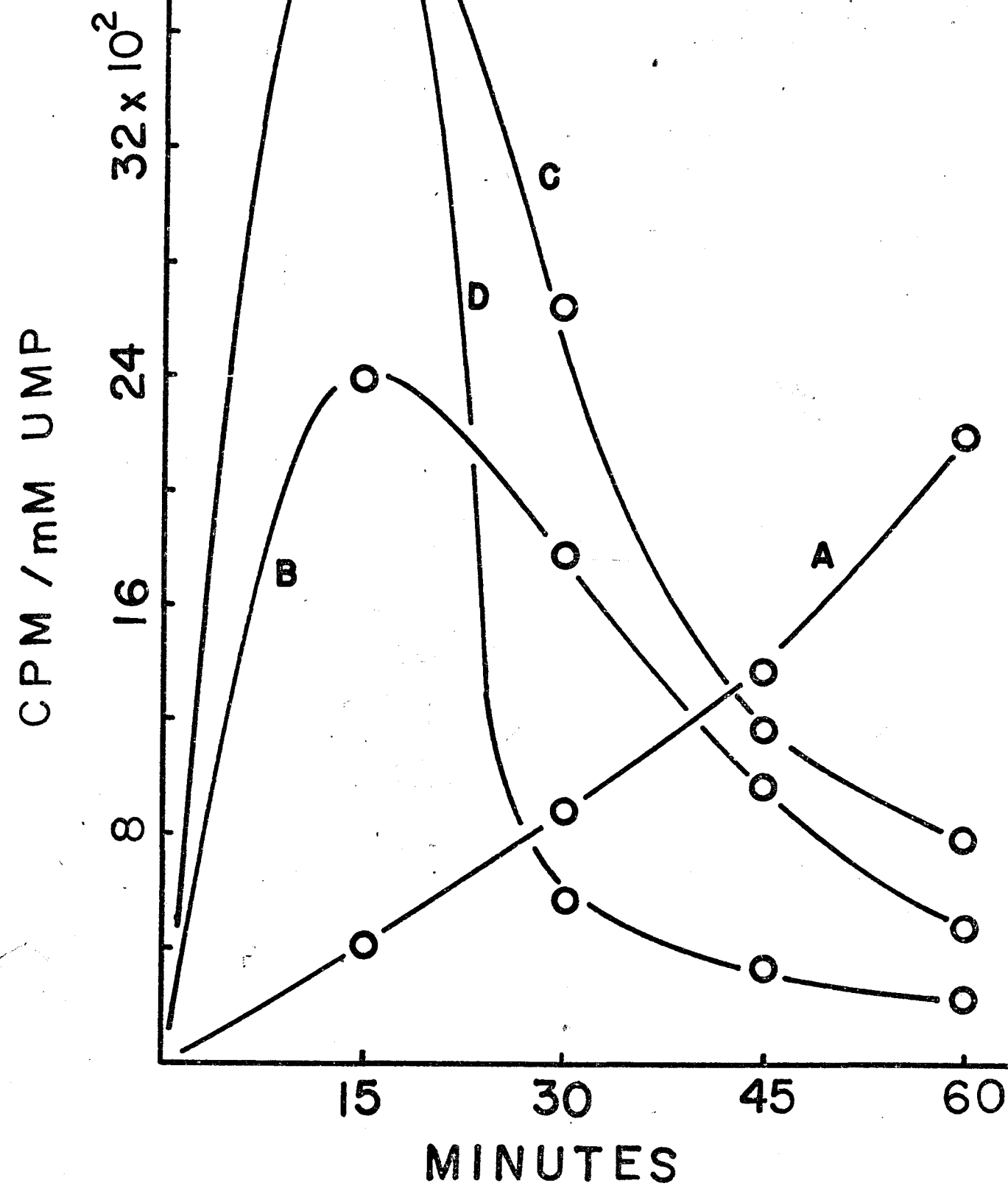


Fig. 16: The rate of formation and turnover of nuclear RNA fractions of Extract 5 of nuclei isolated from wound tissue which has regenerated for 12 days. Extract 5 were extracted at pH 8.6. Symbols as in Fig. 5.

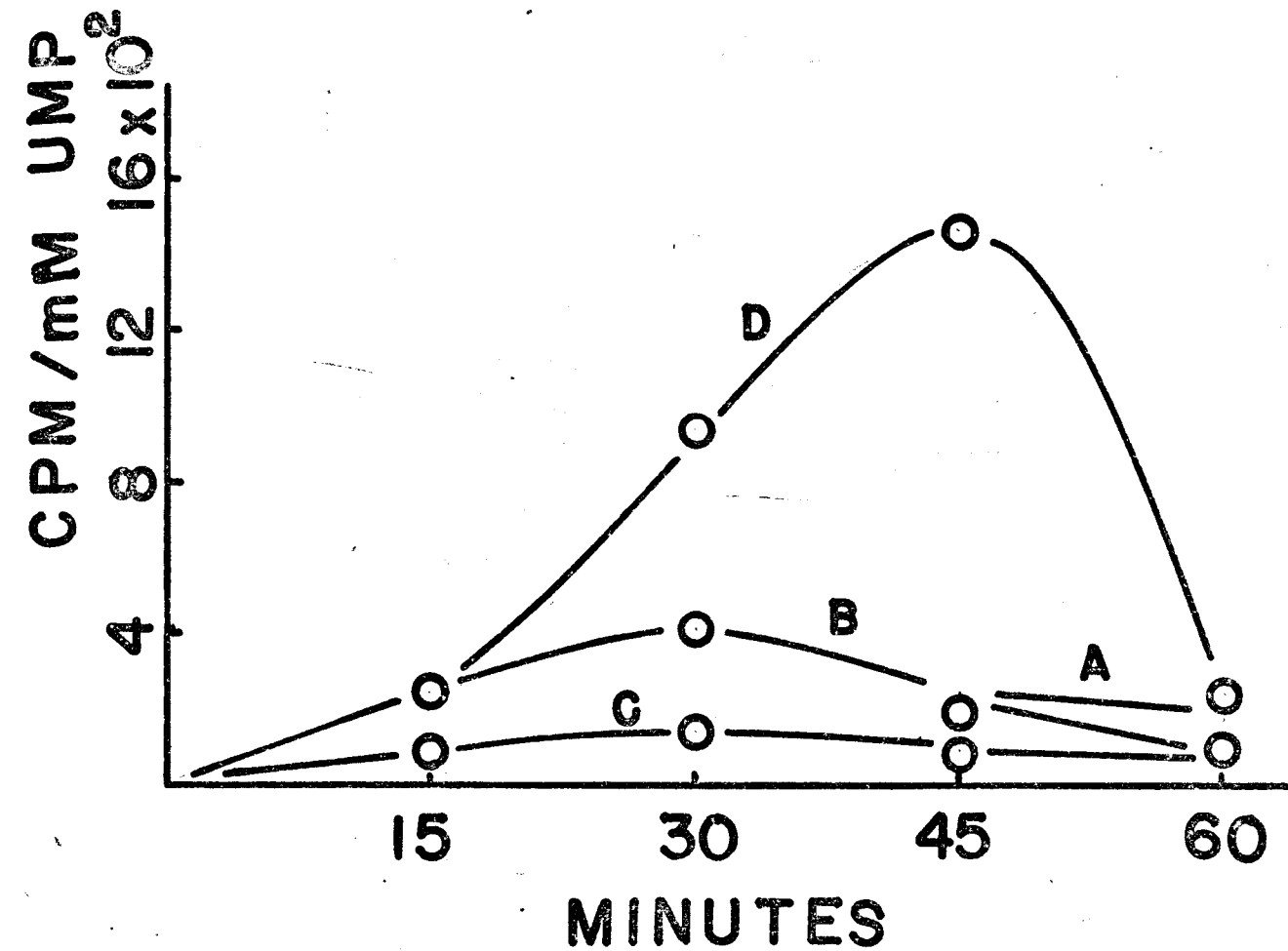


Fig. 17: The rate of formation and turnover of nuclear RNA fractions of Extracts 6-7 of nuclei isolated from wound tissue which has regenerated for 5 days. Extracts 6-7 were extracted at pH 8.6. Symbols as in Fig. 5.

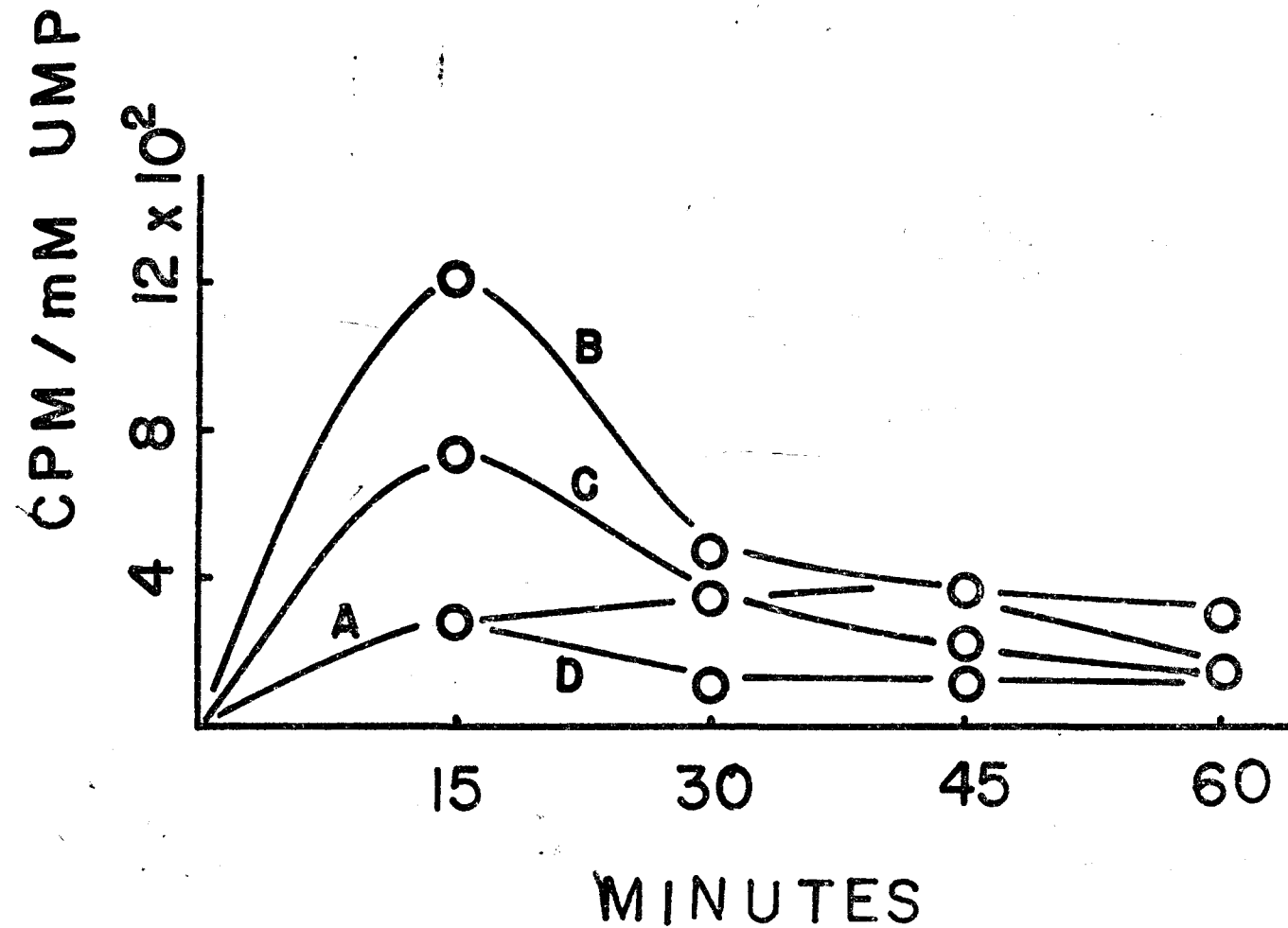


Fig. 18: The rate of formation and turnover of nuclear RNA fractions of Extracts 6-7 of nuclei isolated from wound tissue which has regenerated for 8 days. Extracts 6-7 were extracted at pH 8.6. Symbols as in Fig. 5.

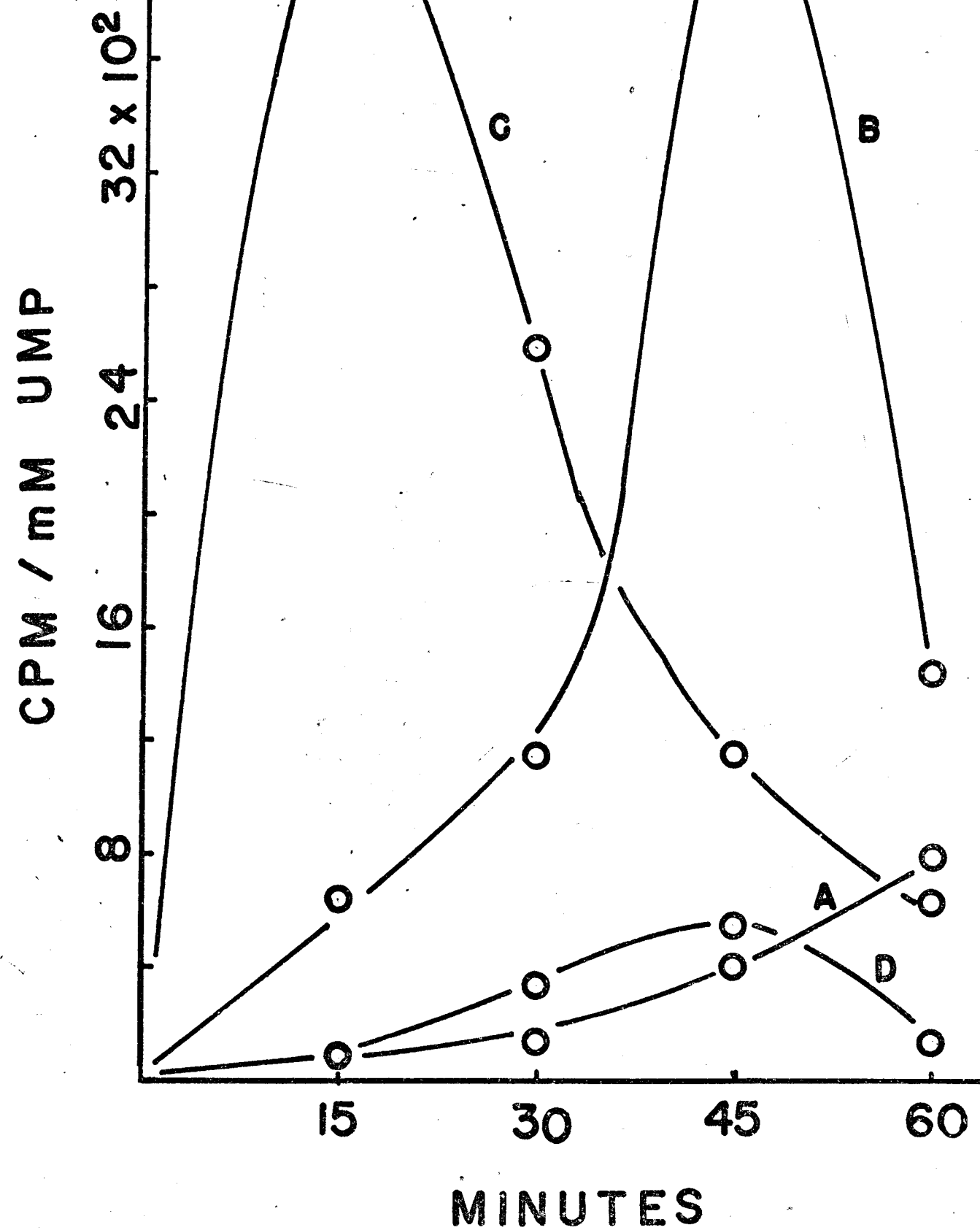
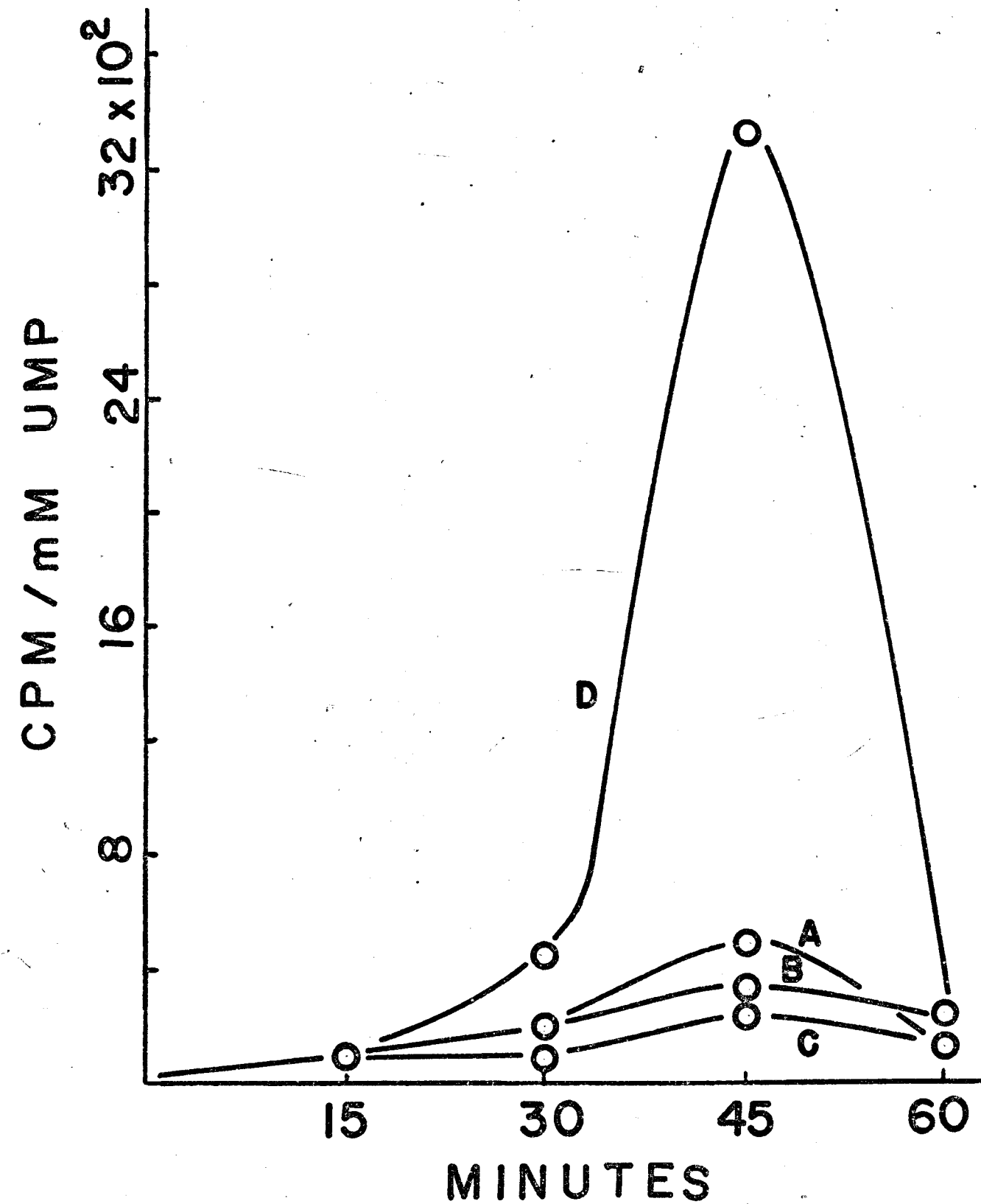


Fig. 19: The rate of formation and turnover of nuclear RNA fractions of Extracts 6-7 of nuclei isolated from wound tissue which has regenerated for 12 days. Extracts 6-7 were extracted at pH 8.6. Symbols as in Fig. 5.



a low G+C/A+U ratio, as regeneration proceeds. The greatest increase in the absolute amount of this low G+C/A+U type(s) of RNA occurs between 5 and 8 days after wounding (Table VII), paralleling the time when the greatest increase in the rate of protein synthesis is observed (134, 282, 283). The content of these DNA-like RNA moieties thereafter remains at about the same level. The percentage of these low base ratio RNA, however, increases steadily (Table VII), under-scoring perhaps the increase in complexity of metabolic activities and greater degree of specialization of the cells, such as polymerization of collagen fibers, contraction and involution of the wound tissue, as the regenerated tissue matures. The absolute amount of RNA with a high G+C/A+U ratio (Table VII and Fig. 20) doubles from the 5th to the 8th day of tissue regeneration; thereafter the amount of such types of RNA decreases markedly. As reported by Williamson et al., (274, 276, 279A), the highest rate of DNA formation and increase in fibroblast population occurred in the wound tissue at about 4 to 5 days after wounding. Baldwin and Rusch (1965) and Gross (1968) have reported that differentiation of cell is characterized by an unusually large accumulation of r-RNA in the nucleus of the cell (15A, 92A). The high percentage of the high G+C/A+U ratio RNA, presumably mostly r-RNA, on the 5th day after wounding (Table VII and Fig. 21), therefore, may indicate that much differentiation of fibroblasts from

TABLE VII

DISTRIBUTION OF NUCLEAR RNA IN REGENERATING
CUTANEOUS TISSUE

G+C/A+U OF RNA	% of RNA			μ M RNA/mg. DNA		
	Days after wounding			Days after wounding		
	5	8	12	5	8	12
1.0	10	31	45	15	96	95
1.4 \pm 0.3	90	69	55	136	212	116

Fig. 20: Relative distribution and characteristics of nuclear RNA per cell in regenerating cutaneous tissue. The height of the bar indicates the mean for 4 pools of 20 rats each. \square represents total nuclear RNA, \blacksquare for RNA molecules with $G+C/A+U$ ratio = 1.4 ± 0.3 , \hline for RNA molecules with $G+C/A+U$ ratio ≤ 1.0 .

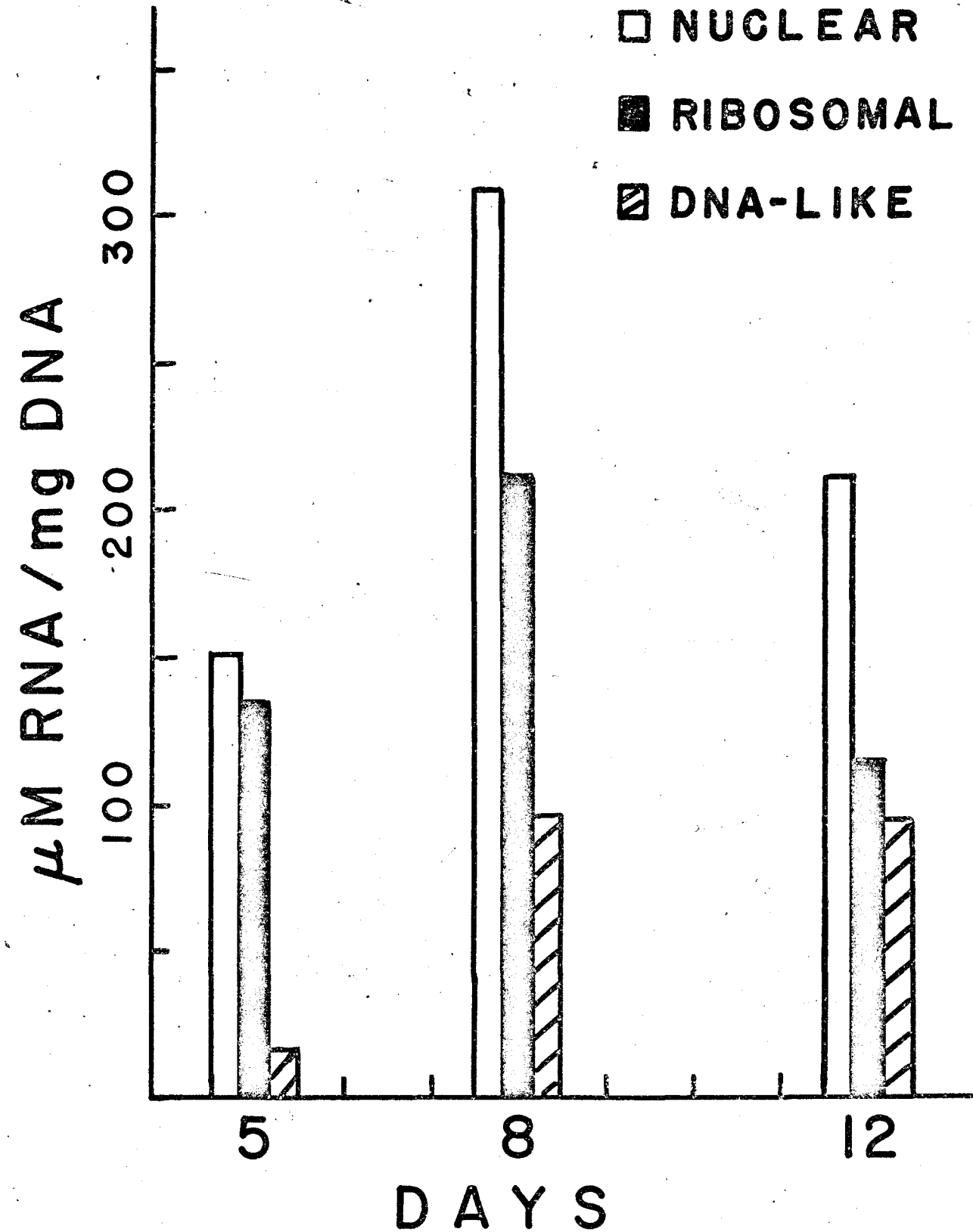
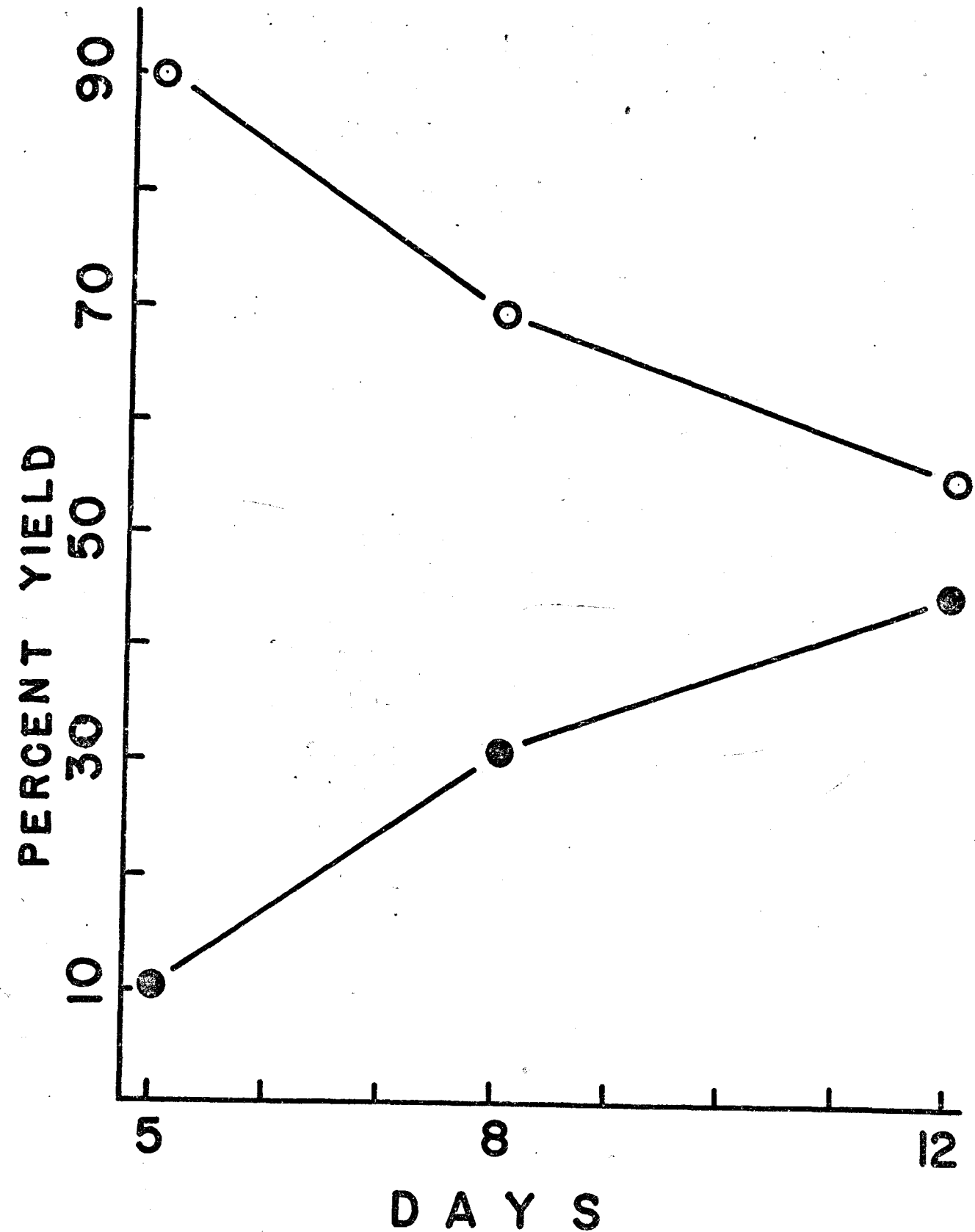


Fig. 21: Relative proportion of various types of nuclear RNA in the wound tissue at different stages of regeneration. \circ represents RNA molecules with G+C/A+U ratio $= 1.4 \pm 0.3$, \bullet for RNA molecules with G+C/A+U ratio ≤ 1.0 .



mesenchymal cells is still taking place at this stage of tissue regeneration. It appears that the level of differentiation decreases as the regenerated tissue matures.

These quantitative and qualitative changes in the nuclear RNA probably indicate that the template activity in the cells of wound tissue varies considerably during the course of regeneration. It remains to be seen whether the quantitative variations in nuclear RNA, may possibly be due to the presence of different levels of RNA polymerase or ribonuclease, or both. The appearance of different types of nuclear RNA during tissue regeneration probably is the result of derepression, followed by transcription, of new and different genomes on DNA, possibly by the same mechanisms as found in liver regeneration (8, 18, 236, 246). The data also seem to suggest that a direct relation exists between the amount of nuclear RNA and the rate of protein synthesis observed in regenerating cutaneous tissue.

RIBONUCLEASE IN REGENERATING WOUND TISSUE

To better understand the changes which occur in the nuclear RNA during tissue regeneration, the effect of ribonuclease on RNA metabolism was studied. The level of active ribonuclease per cell in the wound tissue varies as tissue regeneration progresses (Table VIII and Fig. 22). The level of active RNase was highest on the 5th day after wounding. Following that, it decreased sharply until about the 8th day, when it reached its lowest point during the period studied. From the 8th to the 12th day, the level of active RNase increased gradually. The amount of active RNase on the 12th day was about 85% of that on the 5th day. These variations in the level of the active ribonuclease were almost inversely proportional to the level of RNA in the nuclei of cells in regenerating wound tissue discussed under the previous heading. It therefore appears that changes in the level of nuclear RNA in regenerating wound tissue may be controlled, at least partly, by varying the amount of active ribonuclease per cell; viz. decreasing the level of the active enzyme to effect a rise in the amount of RNA, and vice versa. Results of these studies seem to imply that the level of active RNase plays a significant and effective part in regulating the level of nuclear RNA in a cell.

The amount of total (active + bound) ribonuclease per cell in the wound tissue also varies as tissue regeneration progresses

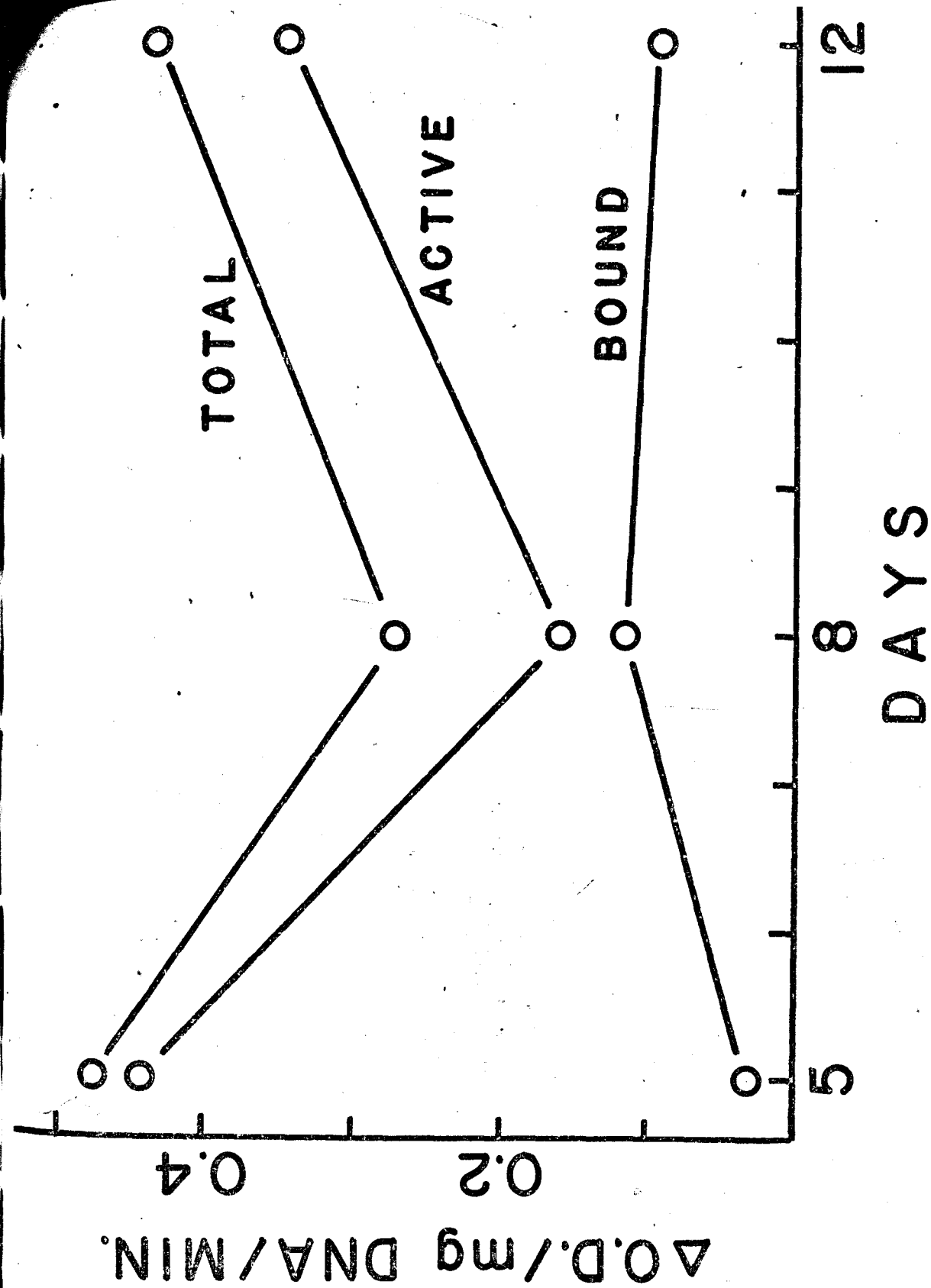
TABLE VIII
RELATIVE LEVEL OF RIBONUCLEASE PER
CELL IN REGENERATING WOUND TISSUE*

RNase	Relative level of enzyme per cell (Δ O.D. _{260 nm} /mg DNA/min.)		
	Days after wounding		
	5	8	12
Total (Active + Bound)	$0.471 \pm 0.072^{**}$	0.276 ± 0.034	0.436 ± 0.014
Active	0.439 ± 0.009	0.158 ± 0.003	0.344 ± 0.003
Bound	0.032	0.118	0.092

*Data is obtained from 3 groups of 3 rats each.

**Mean \pm standard deviation.

Fig. 22: Relative level of various forms of ribonuclease per cell in regenerating cutaneous tissue.



(Table VIII and Fig. 22). The amount was greatest on the 5th day and decreased until about the 8th day. Following that, it increased appreciably, with the level on the 12th day slightly lower than that on the 5th day (Fig. 22). These changes in the level of total RNase during tissue regeneration roughly paralleled that of active RNase. This seems to infer that a decrease in active RNase was brought about partly by a decrease in the amount of total RNase, perhaps due to a decrease in synthesis of RNase by the cell, and vice versa.

As shown by the data in Table IX, the percentage of RNase molecules bound to RNase-inhibiting-protein in a cell increased drastically from the 5th until about the 8th day after wounding. Thereafter, it decreased to a slightly lower level. This raised the question whether this was due solely to the decrease in the total amount of ribonuclease synthesized, without there taking place at the same time any change in the amount of RNase-inhibiting-protein in the cell, hence giving the appearance of an increase in the proportion of bound RNase. Results of the study on the level of bound RNase per cell (Table VIII) indicated that such was not the case. As shown by the data, in conjunction to the over-all decrease in the total amount of RNase molecules synthesized, there was a four-fold increase in the level of RNase-inhibiting-protein from the 5th to the 8th day, followed by a slight decrease thereafter. These studies indicate that

TABLE IXDISTRIBUTION OF RIBONUCLEASE IN
REGENERATING WOUND TISSUE

RNase	% of total RNase		
	Days after wounding		
	5	8	12
Total	100	100	100
Active	93	57	79
Bound	7	43	21

a decrease in the level of active RNase in a cell is brought about by a decreased synthesis of RNase enzyme and also by increasing the level of RNase-inhibiting-protein which further reduces the level of the active enzyme; an increase in active RNase by an increased synthesis of RNase enzyme and a decreased level of RNase-inhibiting-protein. It seems therefore that the regulation of the level of RNase activity in the cell involves a dual control mechanism; i.e. the genetic level which regulates the amount of the enzyme synthesized, and the localized RNase-inhibitor system, perhaps with the former acting as a coarse control and the latter as a fine control as is the case for several other enzymes (247A). It remains to be seen whether such a control mechanism for ribonuclease is an universal one in all living organisms.

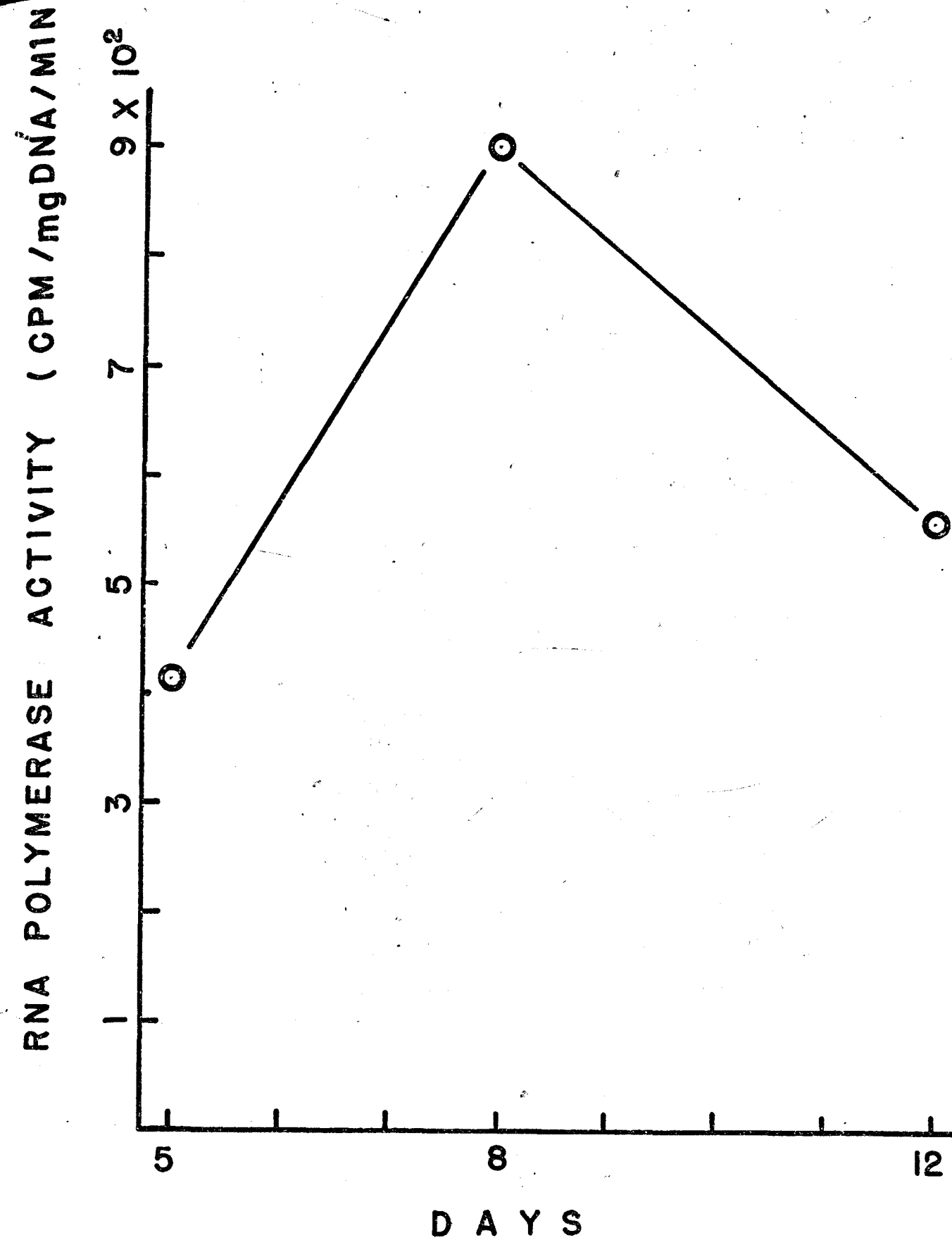
To find out what type of ribonuclease was involved in regulating the amount of nuclear RNA in regenerating wound tissue, aliquots of the supernatant from wound tissue harvested at different stages of regeneration were heated in boiling water-bath for five minutes, followed by assay for RNase activity. We found that the enzyme activity was totally abolished by the heat treatment. This eliminates the possibility of pancreatic RNase being the one involved, since, as mentioned in the introduction, pancreatic RNase is heat stable (21). In our study of RNase activity, the assays were carried out at

pH 7.8, the optimum pH of alkaline RNase. At pH 7.8, acid RNase is inactive. Furthermore, acid RNase is not inhibited by RNase-inhibiting-protein. The RNase in the regenerating wound tissue, as demonstrated by the assays, was readily inhibited by RNase-inhibiting-protein. This inhibition could be removed by reaction with Pb^{++} . Thus it appears that the intracellular alkaline ribonuclease was the main type of RNase involved in the over-all metabolism of nuclear RNA in regenerating wound tissue.

DNA DEPENDENT RNA POLYMERASE IN REGENERATING WOUND TISSUE

DNA dependent RNA polymerase [Nucleosidetriphosphate: RNA nucleotidyl transferase (DNA dependent ; EC 2.7.7.6)] is the enzyme required for the synthesis of all types of cellular RNA in higher organisms, using DNA as the template. In an attempt to unravel some of the factors which may be involved in regulating the metabolism of nuclear RNA during tissue regeneration, we therefore have studied the level of the enzyme in the regenerating wound tissue, using a modified procedure of Bannai et al. (19, 243). Since different regions of the DNA are exposed for transcription at different stages of tissue regeneration, resulting in RNA molecules having different base compositions as shown by our data discussed earlier, in our assay of the level of RNA polymerase, a modification was made whereby aliquots of the same, pooled, stripped rat liver DNA was used as template. As shown by the data in Figure 23, the level of DNA dependent RNA polymerase in the nuclei of cells changes at different stages of tissue regeneration. Of the three stages observed, the relative level of the enzyme per nucleus is lowest on the 5th day after wounding (about 46% of that on the 8th day). Following that it rises rapidly and reaches a maximal level on the 8th day. Thereafter, it decreases gradually. The level of the enzyme on the 12th day is about 60% of that on the 8th day. This variation in the level of DNA dependent RNA polymerase

Fig. 23: Relative level of DNA-dependent RNA polymerase in nuclei of regenerating cutaneous tissue.



corresponds well with the changes in the relative amount of nuclear RNA per cell and the rate of protein synthesis observed in the wound tissue at different stages of regeneration. It appears, therefore, that the amount of nuclear and total RNA per cell, and subsequently the protein synthesizing activity, in regenerating cutaneous tissue is, in part, directly related to and may even be regulated by the level of DNA dependent RNA polymerase in the cell of the wound tissue, instead of a total reliance upon the availability of gene sites for transcription. It remains to be clarified whether an increase in DNA dependent RNA polymerase and an increased number of transcription sites on DNA is a simultaneous happenstance, or that the occurrence of one triggers the other.

HISTONES AND GENE REGULATION IN REGENERATING WOUND TISSUE

As stated earlier in the research proposal, present-day concepts of cellular biology emphasize the role of nucleic acids in directing cellular activities and functions. The necessary information for these cellular processes is contained in the nucleotide sequences of DNA. Thus it appears that the regulatory mechanism of tissue regeneration involves primarily the expression of the appropriate genomes; all the other metabolic processes are merely passive occurrences of the programmed cycle. As a follow-up of our study of the biochemistry of tissue regeneration, we have therefore conducted a study of the mechanisms which may be involved in the expression of the genomes in regenerating cutaneous tissue; viz. the histones and gene derepression.

As shown by the data in Table X, the relative proportion (i.e. the per cent yield) of the lysine-rich to arginine rich histones changes as tissue regeneration progresses. The specific yield of the arginine-rich histones (i.e. mg. histone/mg. DNA), however, does not vary appreciably from the 8th to the 12th day. By contrast, a much greater degree of variation is observed in the lysine-rich histones during the same experimental periods. This is probably due to the very-lysine-rich histones of the lysine-rich histone fraction whose proportions had been reported to vary greatly in developing tissues

TABLE X

DISTRIBUTION OF HISTONE IN REGENERATING WOUND TISSUE

Days after wounding	mg Histone/mg DNA			% yield of Total	
	Lysine-rich	Arginine-rich	Total	Lysine-rich	Arginine-rich
5	3.755	0.431	4.186	89.9	10.2
8	1.522	0.224	1.746	87.2	12.8
12	1.206	0.242	1.448	83.3	16.7

(72, 104, 236). It appears from these observations, that the metabolism of the histones in regenerating cutaneous tissue does not differ to any appreciable extent from that in the regenerating liver.

Table X also shows that the relative content (i.e. mg histone/mg DNA) of the histones on the 5th day after wounding is about twice as much as those on the 8th and 12th days. The per cent yields of the lysine- and arginine-rich histones in the cell on the 5th day, however, remain within the range of those observed on the other days. This finding of an elevated relative content of both lysine- and arginine-rich histones per cell occurred at a time when the highest rate of DNA formation and increase in fibroblast population were observed (95, 276); viz. during a time of high mitotic activity. The data therefore seem to suggest that during cell replication, the synthesis and accumulation of histones precedes the synthesis of DNA. Such a phenomenon has also been observed in regenerating liver (58, 236).

As mentioned earlier, it has been reported that gene derepression, in higher organisms, involves the methylation, phosphorylation and/or acetylation of various histones. As shown in Table XI, the methylation, phosphorylation and acetylation of both the lysine- and arginine-rich histones can be readily detected in regenerating cutaneous tissue. The degree of sub-

TABLE XI

RELATIVE DEGREE OF SUBSTITUTION OF HISTONES IN REGENERATING
CUTANEOUS TISSUE

Days after wounding	CPM/mg. Histone					
	Lysine-rich Histone			Arginine-rich Histone		
	$C^3H_3^+$ labelled	$^{32}PO_4^{\equiv}$ labelled	$C^3H_3-\overset{O}{\parallel}C-O^-$ labelled	$C^3H_3^+$ labelled	$^{32}PO_4^{\equiv}$ labelled	$C^3H_3-\overset{O}{\parallel}C-O^-$ labelled
5	2419	698	45	220	406	32
8	1236	1683	13	152	577	95
12	445	2097	29	124	388	67

stitution by each chemical group, however, varies at different stages of tissue regeneration, and also from histone to histone.

In contrast to regenerating liver where only the arginine-rich histones were methylated, we found that in regenerating cutaneous tissue, both the lysine- and arginine-rich histones were methylated (Table XI). The specific activity of the lysine-rich histones in all cases were actually much greater than that of the arginine-rich histones. The extent of methylation of both the lysine- and arginine-rich histones were highest on the 5th day after wounding, and decreased sharply thereafter. Tidwell et al. have reported that the methylation of lysine- and arginine-rich histones is associated with cell differentiation (236, 246). As pointed out earlier in the discussion of nuclear RNA metabolism, it appears that much differentiation of fibroblasts from mesenchymal cells is still taking place in the wound tissue on the 5th day after wounding. The level of differentiation decreases as the regenerated tissue matures. It therefore appears that the methylation of the histones in the regenerating cutaneous tissue may be related to cell differentiation activities, as in the case of the regenerating liver.

As shown in Table XI, both the lysine- and arginine-rich histones are phosphorylated during tissue regeneration. The extent of phosphorylation of the lysine-rich histones increases

as the regenerated tissue matures. Phosphorylation of the lysine-rich histones has been reported to be inversely related to the degree of mitotic activity of the tissue (98, 163, 236). Williamson et al. (274, 276), had reported that the mitotic activity in the regenerating cutaneous tissue was highest on the 5th day after wounding, and decreased markedly thereafter. These observations seem to suggest that the phosphorylation of lysine-rich histones in regenerating cutaneous tissue, may also be related to the regulation of the mitotic activities of the cell, as in the case of the regenerating liver.

The arginine-rich histones have been reported to prefer regions of DNA rich in G and C residues (114, 236). The extent of phosphorylation of the arginine-rich histones during cutaneous tissue regeneration (Table XI) appears to coincide with the changes in the relative level of the high G+C/A+U ratio RNA in the nuclei of cells of wound tissue at different stages of regeneration (Figure 20). However, since similar observation has not been reported in the literature, it is unclear at the moment whether any correlation can be drawn between the two.

Acetylation of the arginine-rich histones has been reported to increase RNA synthesis in the regenerating liver (236). The relative degree of acetylation of this fraction of histones in the regenerating cutaneous tissue (Table XI) seems to

parallel the relative amount of r-RNA and total nuclear RNA per cell. Acetylation of the lysine-rich histones, as shown in Table XI, can be readily detected in the regenerating cutaneous tissue. At this juncture, there appears to be no discernable relation between the acetylation of the lysine-rich histones and the mitotic activity of cells in regenerating cutaneous tissue. However, the extent of acetylation of the lysine-rich histones appears to parallel the relative level of total ribonuclease in the cell (Figure 22) during tissue regeneration.

Chapter VI

SUMMARY AND CONCLUSIONS

Regenerating wound tissues were harvested from adult female rats after the subcutaneous administration of 5-³H-Uridine, on the 5th, 8th and 12th day after wounding. The tissue samples were homogenized and the nuclei were isolated by sucrose gradient centrifugation. RNA was extracted from the lysed nuclei suspended in aqueous phenol by successive volumes of neutral and alkaline Tris buffer. The RNA in each extract was precipitated with ethanol, and then fractionated on a ECTEOLA cellulose column by stepwise gradient elution with LiCl solution. The absorption spectrum of each RNA fraction at pH 7.2 in the UV region was recorded.

After mild alkaline hydrolysis, the bases in each nuclear RNA fraction were separated on a Dowex 50W-X⁴ column. Concentration of each nucleotide, and subsequently the G+C/A+U ratio of each RNA fraction, were calculated from the spectrophotometric data.

After spectrophotometric determination, the activity of UMP was assessed by liquid scintillation counting. The rate of turnover of each nuclear RNA fraction was obtained by plotting specific activity of UMP against minutes after injection of 5-³H-Uridine.

The amount of DNA in each tissue sample was determined by

reaction with diphenylamine.

To measure the level of RNase during tissue regeneration, the tissue samples were homogenized in Tris buffer at pH 7.8. After centrifugation, the level of active RNase in the sample was measured by incubating aliquots of the supernatant with RNA at 37°C. Undigested RNA was removed by ethanol precipitation, and the optical density of the soluble fraction was determined. The level of total RNase, (i.e. both the active and bound RNase), was determined by treating aliquots of the supernatant with lead acetate, and then assaying them for RNase activity as in the case of the active RNase.

The relative level of DNA-dependent RNA polymerase in the regenerating cutaneous tissue was measured using a modified procedure of Teng et al. The regenerating tissue samples were homogenized and the nuclei were isolated. Aliquots of the ruptured nuclei suspension was incubated with stripped rat liver DNA template and a volume of the substrate, with 5-³H-UTP as the tracer. Unincorporated trinucleotides were then removed by ethanol precipitation, and centrifugation. The radioactivity of the incorporated nucleotides in each RNA sample was then determined by liquid scintillation counting. The specific activity of each sample was taken as a relative measure of the amount of DNA-dependent RNA polymerase present in the tissue.

In the third phase of this study, an attempt was made to study the mechanisms of gene-derepression involved during tissue regeneration.

Regenerating wound tissues were harvested from groups of rats on the 5th, 8th and 12th day after wounding, following the administration of L-(methyl- ^3H)-Methionine and $^{32}\text{PO}_4$. In an identical batch of wounded rats, the tissue samples were similarly harvested following the subcutaneous injection of $\text{C}^3\text{H}_3-\overset{\text{O}}{\underset{\text{O}}{\text{C}}}-\text{O}^-$.

Lysine-rich histones and arginine-rich histones were then isolated from the cell nuclei of these regenerated tissues by citric acid and very dilute hydrochloric acid extraction, followed by acetone precipitation. After purification by Bio-gel column chromatography, the amount of each histone fraction was determined. The radioactivity of each isotope in the respective histone fractions was then measured by liquid scintillation counting.

From the results of this study, it was found that the level of total nuclear RNA per cell, increases rapidly to a maximal level in about 8 days after wounding, and then begins to decline. A comparison of the amount of RNA in various cell compartments of regenerating cutaneous tissue indicates that the changes in RNA content as tissue regeneration proceeds are due mainly to changes in the amount of RNA in the cell nuclei.

Judging from the spectral and the nucleotide analysis data, nuclear RNA metabolism in the wound tissue seems to entail a steady increase in the amount and types of DNA-like RNA having a low G+C/A+U ratio, as regeneration progresses. The greatest increase in the absolute amount of this low G+C/A+U types of RNA occurs between 5 and 8 days after wounding, paralleling the time when the greatest increase in the rate of protein synthesis is observed. The content of these DNA-like RNA moieties thereafter remains at about the same level. The percentage of these low base ratio RNA in the nuclei, however, increases steadily, under-scoring perhaps the increase in complexity of metabolic activities and greater degree of specialization of the cells, such as polymerization of collagen fibers, contraction and involution of the wound tissue, as the regenerated tissue matures.

The absolute amount of nuclear RNA with a high G+C/A+U ratio increases two-fold from the 5th to the 8th day of tissue regeneration; thereafter, the amount of such type(s) of RNA decreases markedly. As reported by Williamson et al. (274, 276, 279A), the highest rate of DNA formation and increase in fibroblast population occurred in the wound tissue at about 4 to 5 days after wounding. Gross et al. (15A, 92A), had reported that differentiation of cell is characterized by an unusually large accumulation of r-RNA in the nucleus. The

large percentage of the high G+C/A+U ratio RNA, presumably mostly r-RNA, on the 5th day after wounding may therefore indicate that much differentiation of fibroblasts from mesenchymal cells is still taking place at this stage of tissue regeneration. It appears that the level of cell differentiation decreases as the regenerated tissue matures. These quantitative and qualitative changes in the nuclear RNA probably indicate that the template activity in the cells of wound tissue varies considerably during the course of regeneration.

The level of DNA-dependent RNA polymerase in the nuclei of cells changes as tissue regeneration progresses. The variations in the level of this enzyme correspond well with the changes in the relative amount of nuclear RNA per cell, and the rate of protein synthesis observed, in the wound tissue at different stages of regeneration. The level of active RNase per cell during tissue regeneration also varies. In contrast to DNA-dependent RNA polymerase, the variations in the level of active RNase, however, bear an inverse relation to the level of nuclear RNA in the cells of regenerating wound tissue. These results seem to infer that the variations in the level of nuclear and total RNA per cell, and subsequently the protein synthesizing activity, in regenerating cutaneous tissue are in part due to, or are regulated by, the changes in the levels of DNA-dependent RNA polymerase and active RNase in the cell.

The type of RNase enzyme involved in the over-all metabolism of RNA in regenerating cutaneous tissue, we found, is mainly the intracellular alkaline ribonuclease. The amount of total RNase in the wound tissue follows about the same course as active RNase. The variations in the amount of RNase molecules bound to RNase-inhibiting-protein, however, bear an inverse relation to that of the RNase activity in the cells of regenerating wound tissue. The findings seem to indicate that in regenerating cutaneous tissue, the level of active RNase in the cell may be regulated by a dual control mechanism; viz. the genetic level which regulates the amount of the enzyme synthesized, and the localized RNase-inhibiting-protein system. The former may act as a coarse control and the latter as a fine control, as in the case for several other enzymes.

The relative proportion of the lysine-rich to arginine-rich histones in the wound tissue changes as tissue regeneration progresses. The extent of methylation, phosphorylation and acetylation of the histones varies at different stages of tissue regeneration, and also from histone to histone. These observations indicate that the template activity in the cells of wound tissue varies considerably during the course of regeneration, as was succinctly illustrated by the tremendous qualitative and quantitative changes which occurred in the nuclear RNA.

Despite the large differences between the liver and the

regenerating cutaneous tissue, the mechanisms of gene de-repression in both tissues appear to be quite similar. However, in contrast to regenerating liver where only the arginine-rich histones were methylated, both the arginine-rich and lysine-rich histones were found to be methylated in the regenerating cutaneous tissue. The data also suggest the possibility that during cell replication, the synthesis and accumulation of histones may precede the synthesis of DNA.

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Fig. 24: Standard curve of GMP, expressed as absorbance at 257 nm vs. concentration of GMP.

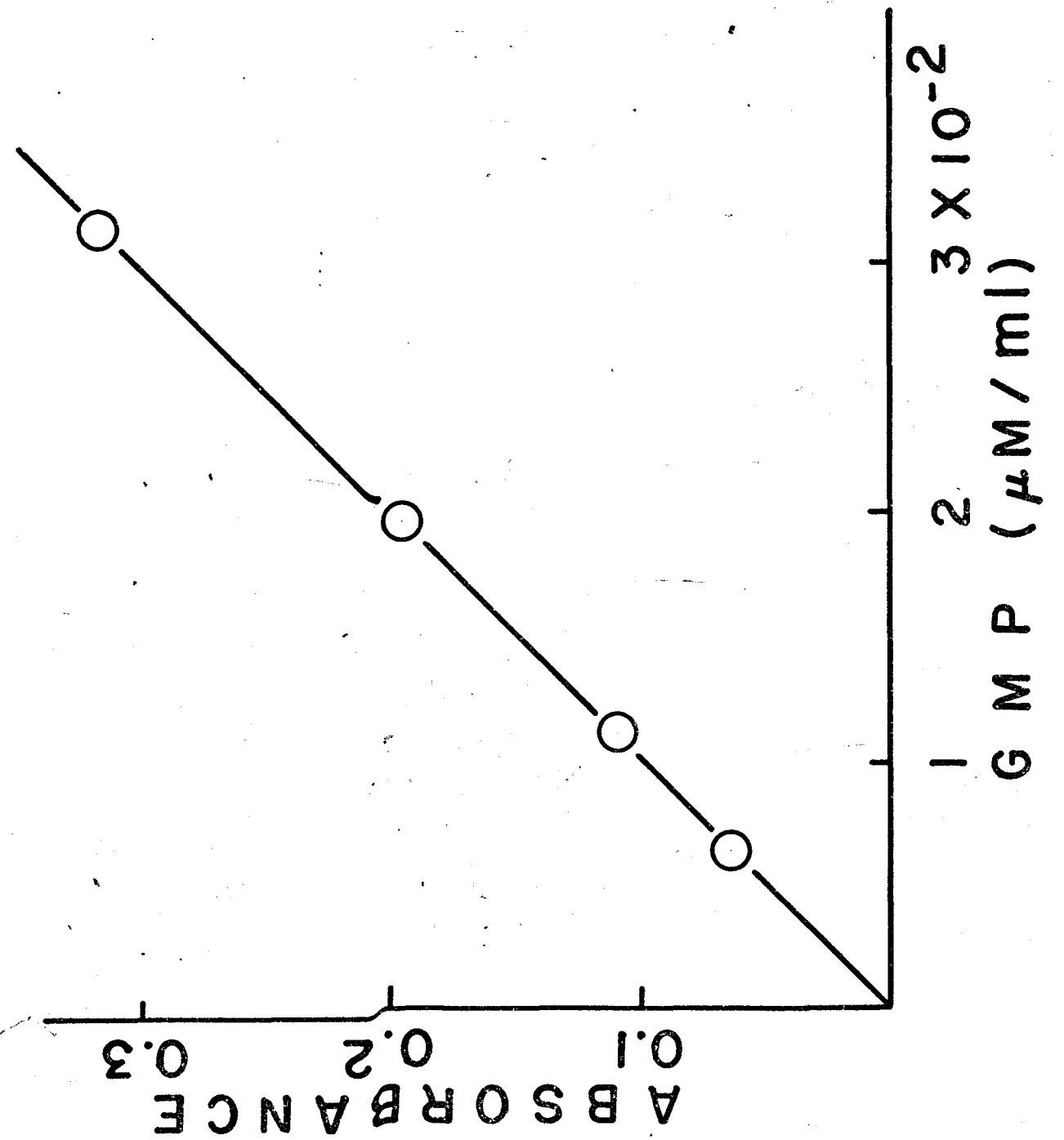


Fig. 25: Standard curve of UMP, expressed as absorbance at 260 nm. vs. concentration of UMP.

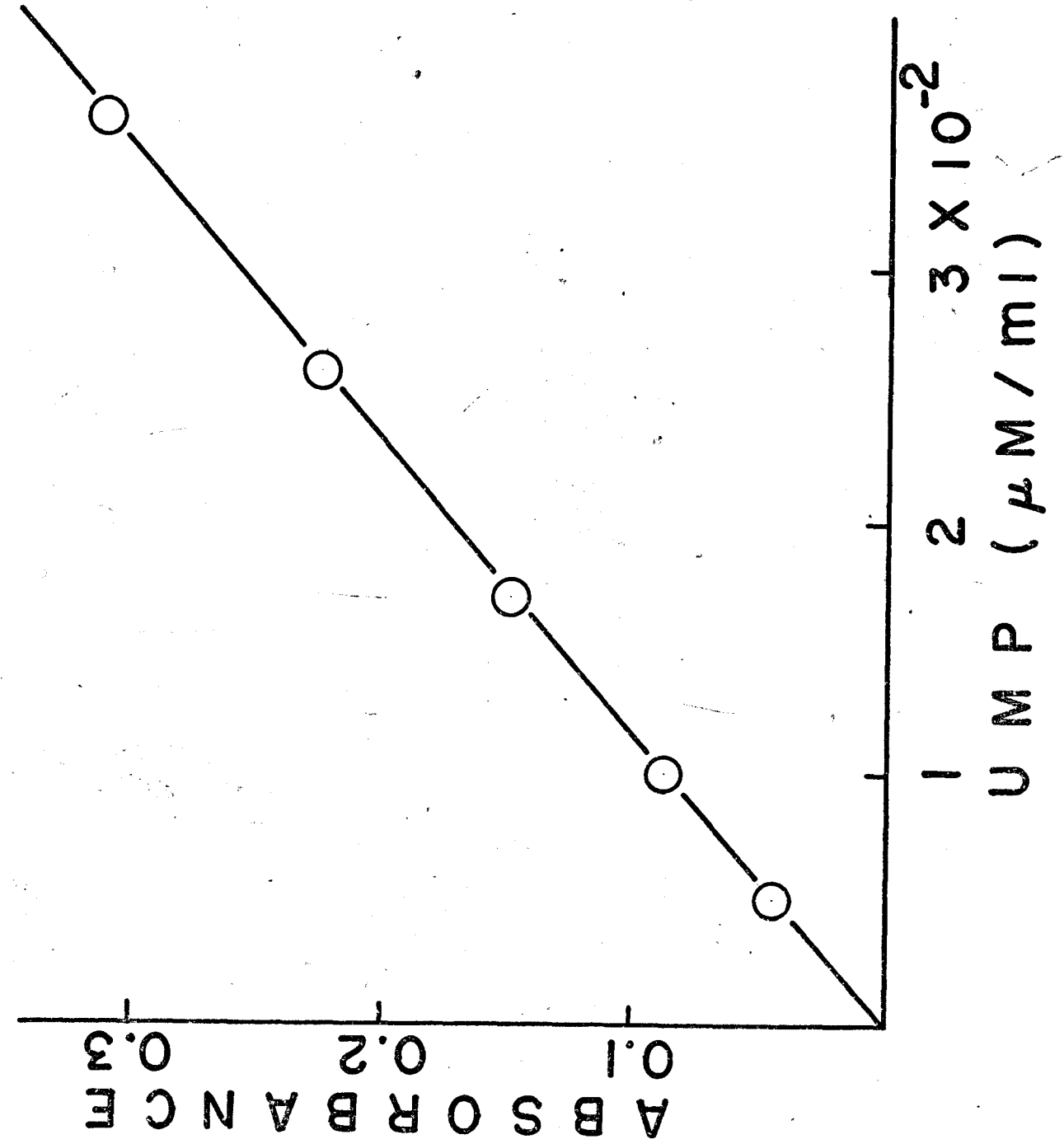


Fig. 26: Standard curve of AMP, expressed as absorbance at 257 nm vs. concentration of AMP.

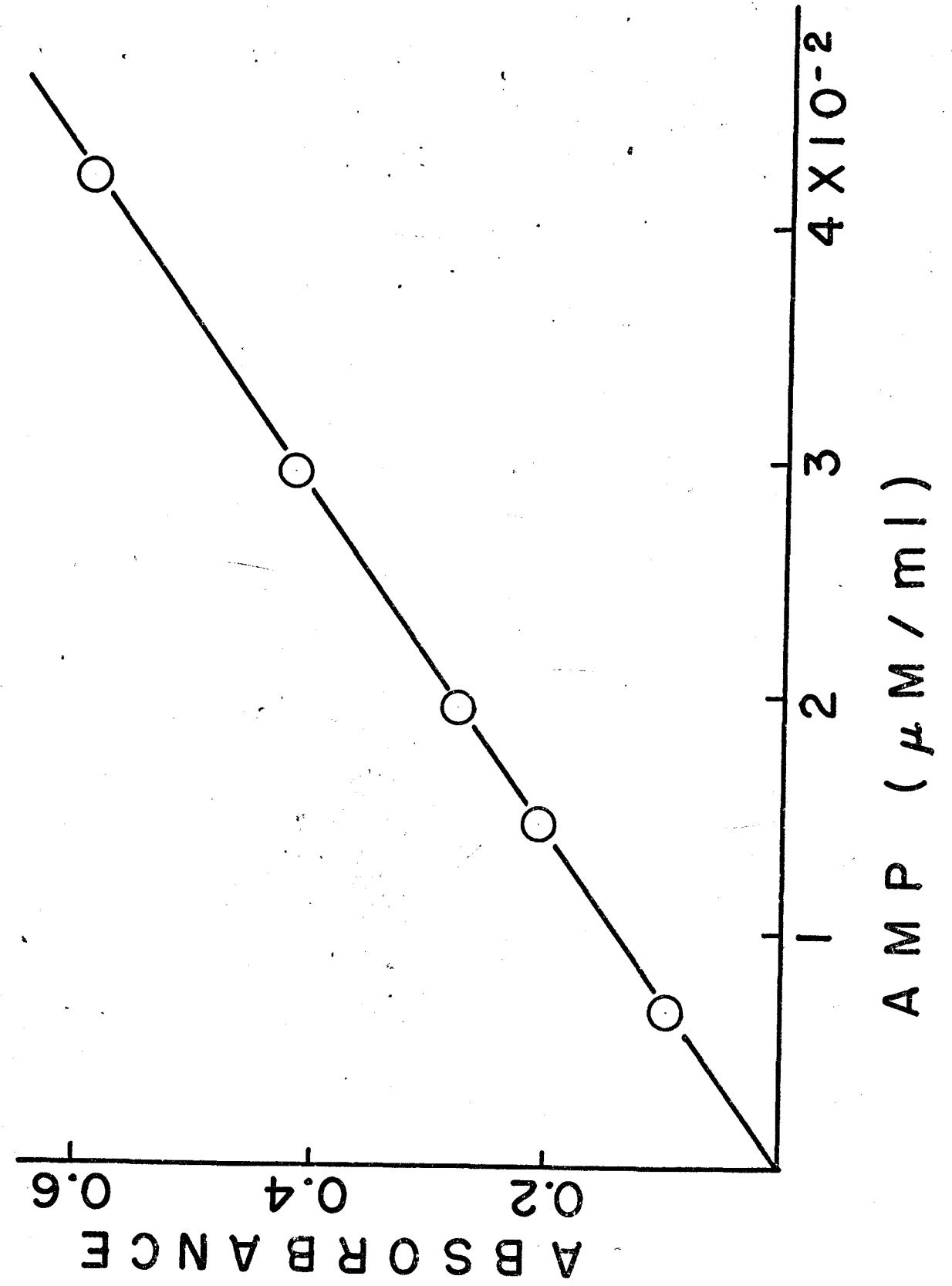
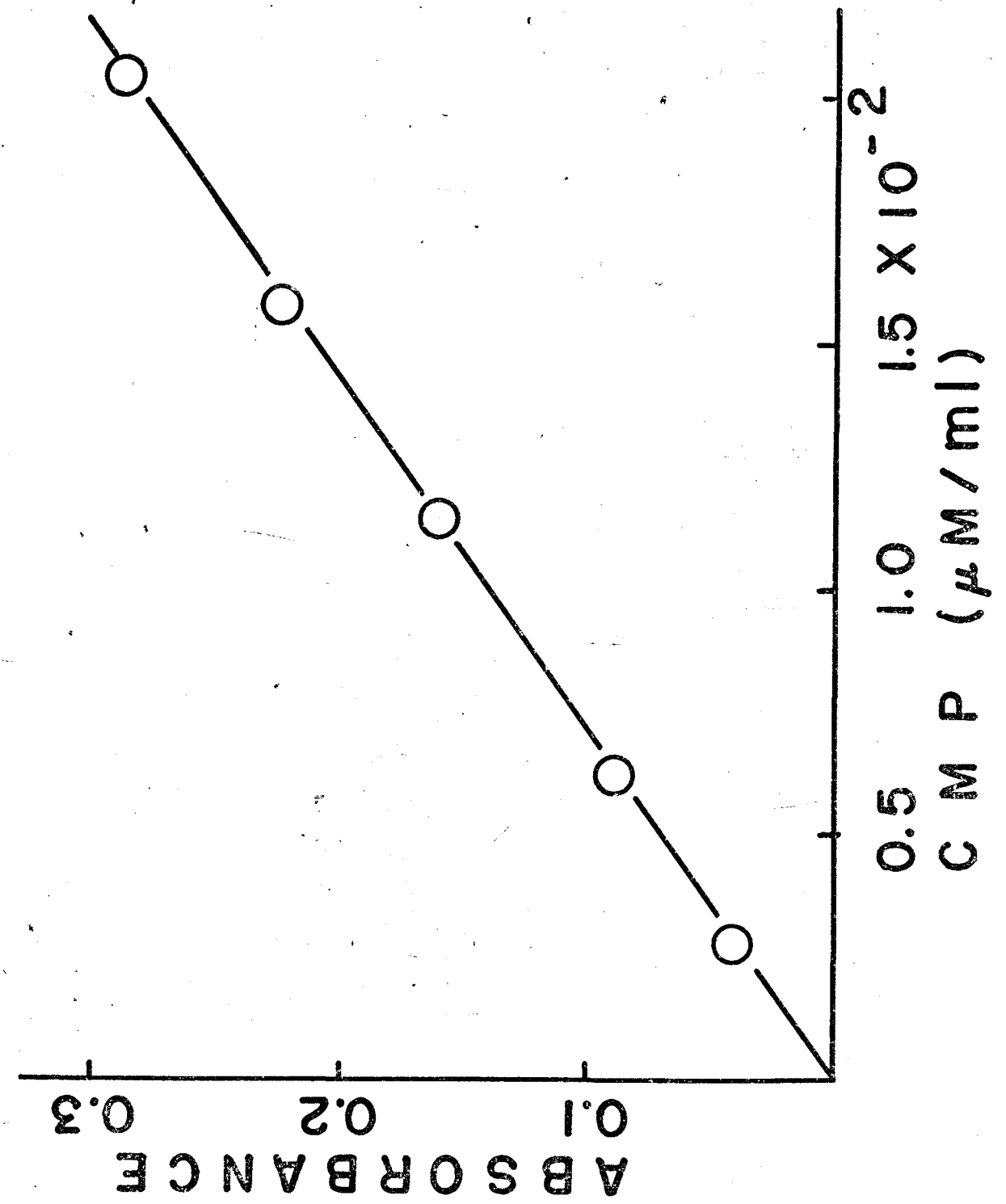


Fig. 27: Standard curve of CMP, expressed as absorbance at 279 nm vs. concentration of CMP.



APPROVAL SHEET

The dissertation submitted by Leong-Ging Wong has been read and approved by five members of the faculty of the Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval with reference to content, form, and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 24, 1971

Date

Maurice J. P. Heuser

Signature of advisor